Studies on Groundnut rosette disease in Ghana and genomic analysis of a novel Phasey bean virus in Australia

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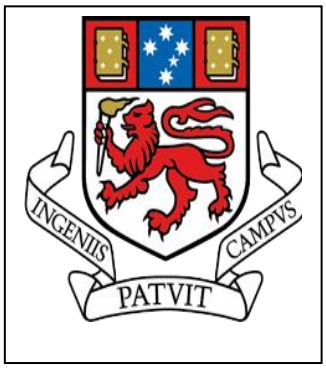
B. Sc. (Hons), M. Phil

Submitted in fulfilment of the requirements
For the degree of

Doctor of Philosophy



UNIVERSITY OF TASMANIA

February, 2017

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my primary supervisor, A/Prof. Calum Wilson (University of Tasmania) and Co-supervisors Dr Robert Tegg (University of Tasmania) and Prof. Samuel Kwame Offei (University of Ghana) for their support, patience and insightful comments which led to significant improvements of this thesis. I am so grateful to you for steering me in the right direction to make this thesis a reality. I could not have imagined having better advisors and mentors for my PhD study.

My sincere thanks also go to Mrs Annabel Wilson, Dr Tamil Thangavel (Tasmanian Institute of Agriculture) Dr Alison Dann, Mr Shane Hossel and Mr Peter Cross (Department of Primary Industries, Parks, Water and Environment) for their immense contribution towards the execution of the research. I am also grateful to Dr Samuel Amiteye, Mr Jonathan Amponsah and Mr Iddriss Mohammed (BNARI, Ghana Atomic Energy Commission) for their assistance with field work conducted in Ghana. I thank my fellow lab mates at the New Town research Laboratories (Mark Balendres, Kritika Krishnamorthy and Sabine Tanois) for the stimulating discussions, and for sharing our coffee breaks and lunch times together.

I am grateful to the Australian Government for the AusAID scholarship. This has been a great opportunity for me to further my education. The generous offer has adequately empowered me to contribute my quota to the development of Agriculture in Ghana. I am also thankful to the Ghana Atomic Energy Commission for granting me study leave with pay and for supporting my field work which was conducted in Ghana.

I would like to express my profound gratitude to my wife (Vivian), children (Carlis, Thelma and Caitlyn) for their unfailing support, continuous encouragement and prayers throughout my years of study. My sincere thanks also go to my parents, brothers and sisters for their support and prayers.

Finally, I would like to thank the Almighty God for His protection and guidance. Without Him, this accomplishment would not have been possible.

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Paper 1: Appiah, A. S., Offei, S. K., Tegg, R. S., and Wilson, C. R. (2016). Impact of groundnut rosette disease on nutritive value and elemental composition of four varieties of peanut (Arachis hypogaea). *Annals of Applied Biology* 168: 400 – 408 **(Chapter 3).**

Paper 2: Appiah, A. S., Offei, S. K., Tegg, R. S., and Wilson, C. R. (2016). Varietal response to groundnut rosette disease and the first report of Groundnut ringspot virus in Ghana. *Plant Disease* 100:946-952 **(Chapter 4)**

Paper 3: Andrew S. Appiah, Frederick L. Sossah, Robert S. Tegg, Samuel K. Offei, Calum R. Wilson (2017). Assessing sequence diversity of Groundnut rosette disease agents and the distribution of Groundnut rosette assistor virus in major groundnut-producing regions of Ghana. *Tropical Plant Pathology* 42:109–120 **(Chapter 5)**

Paper 4: A.S. Appiah, R.S. Tegg, M. Sharman and C.R. Wilson (2017). Host range, complete genome sequencing and molecular phylogeny of a novel Polerovirus from Australian legumes. Manuscript under preparation. **(Chapter 6)**

Conference presentations

Appiah, A.S., Tegg, R.S., Wilson, C.R. Studies on Groundnut rosette disease and other legume viruses. Africa Australia Research Forum on Mining, agriculture and development: Bread from Stones? A joint conference of The Crawford Fund and the Africa Australia Research Forum. 25th - 28th August 2013, Perth, Australia.

Appiah, A.S., Tegg, R.S., Wilson, C.R. Host range, genome sequencing and molecular phylogeny of a novel Polerovirus from Australian legumes. Australasian Plant Pathology Society (Tasmanian Division) 2016 Symposium, 29th April, 2016, Ross, Tasmania, Australia.

Andrew S. Appiah, Robert Tegg, Samuel K. Offei, Calum R. Wilson. Studies on Groundnut rosette disease and implications of the newly reported Groundnut ringspot virus for groundnut production in Ghana. American Phytopathological Society's Annual Conference 'Science to practice', 30th July to 3rd August, 2016, Tampa, Florida, USA

Candidate was the primary author of all papers, contributed to the conception and design of the study and executed field and laboratory work. Candidate also contributed to the analysis and interpretation of data and drafted significant portions of the paper, revision and final approval of all articles.

Robert Tegg contributed to the design of the experiment, guided statistical analysis and interpretation of data and revision and final approval of the manuscripts.

Samuel Offei contributed to the conception of of the research, revision and final approval of the manuscript.

Calum Wilson contributed to the conception and design of the research project, guided statistical analysis and interpretation of results, provided technical support, revision and final approval of all manuscripts.

Frederick Leo Sossah assisted with data collection and analyis and the final approval of the manuscript for Chapter five.

Murray Sharman provided the Queensland isolate of the PhBMYV and the *Aphis craccivora* vector used in the study (Chapter six) and a contributed to the revision and final approval of the manuscript.

Declaration of Agreement

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ABSTRACT

Viruses present a major challenge to the production of major food crops worldwide, including legumes. The diseases they cause have profound effects on both plant growth and the quality of produce, resulting in significant losses. The current study investigated the proximate and elemental composition of four groundnut (peanut, *Arachis hypogaea* L.) cultivars infected with groundnut rosette disease (GRD), screened local cultivars of groundnut for resistance to GRD, detected *Groundnut ringspot virus* for the first time in Ghana and assessed the genetic diversity within Ghanaian isolates of *Groundnut rosette assistor virus* (GRAV), *Groundnut rosette virus* (GRV) and satellite RNA of GRV and compare those with known isolates from other African countires. In a related study, the complete genome of an isolate of novel virus infecting Phasey bean (*Macroptilium lathyroides* L.) in Australia; Phasey bean mild yellows virus (PhBMYV) was sequenced with evidence of genomic recombination found and its transmission to other legumes demonstrated.

Proximate analysis of seeds from GRD-infected groundnuts showed a decrease in moisture and ash content, while fat and energy content increased. Protein and carbohydrate content varied inconsistently between seeds of diseased and healthy plants of the different cultivars. Instrumental neutron activation analysis (INAA) of ten elements within leaves, stems and seeds revealed elevated levels of K, Al and Cl in leaves, stems and seeds in at least three of the four GRD-infected cultivars while Na was decreased in stems but increased in seeds. Despite significant differences, Mg, Mn, Ca and Zn did not show any consistent change with respect to plant part or genotype, between diseased and healthy plants. V and Fe were not detected in seeds but were found at low levels in leaves and stems. This work has been published in Annals of Applied Biology (2015) and represents the first report on the effect of GRD on the nutritive quality of groundnuts.

Twelve cultivars of groundnut were screened in field trials for resistance to GRD in the coastal savannah agro-ecological zone of Ghana. Cultivar 'Oboshie' was rated as highly resistant; 'Bremaowuo', 'Nkatefufuo', and 'Behenase' as resistant; and 'Nkosuor', 'Kumawu', and 'Otuhia' as moderately resistant. GRAV infection rates of 11.8 to 61.8% and 13.9 to 100% were found within the field trial for dry and wet seasons respectively. These included symptomless plants suggesting that some lacked co-infection with GRV and its satellite RNA which are responsible for symptom induction. Some plants exhibited chlorotic and line pattern symptoms suggestive of *Groundnut ringspot virus* (GRSV) infection, which was confirmed by enzyme-linked immunosorbent assay, reverse-transcription polymerase chain reaction, and amplicon sequencing. This represents the first report of GRSV in Ghana. GRSV infection rates within the field trial were 0.0 to 69.5% (dry season) and 26.1 to 69.5% (wet season) and was commonly found in mixed infections with GRAV in all cultivars except Nkosuor and Bremaowuo in the dry season. Graft-inoculated groundnut cultivars showed significantly reduced height, leaf area, chlorophyll content, dry haulm weight, and seed yield compared to healthy plants. The sources of resistance to GRV and possibly GRAV and GRSV identified in this study could be exploited in groundnut breeding programs. This work has been published in Plant Disease (2015).

GRAV incidence in farmers' fields was assessed through crop surveys in the three northern groundnut-producing regions of Ghana. High (69.5 to 75.0%) but insignificantly different incidences were found between the regions. Isolates of GRAV, GRV and sat RNA collected during the survey were sequenced. There were no obvious isolate diversity patterns among the Ghanaian isolates of all three agents of GRD based on the regions from where they were collected. Nucleotide sequences of the coat protein gene of GRAV showed 99-100% identity among the Ghanaian isolates and 97-100% similarity to GRAV sequences from Nigeria and Malawi for both nucleotide and predicted amino acids. Ghanaian GRV isolates

were closer in nucleotide sequence identity to Nigerian isolates (95 - 98%) than Malawian isolates (88 - 90%). Similarly, Ghanaian satRNA isolates shared close nucleotide identities (94-100%), but were distinct from Nigerian (82 - 87%) and Malawian (82 - 86%) isolates. This work has been accepted for publication in Tropical Plant Pathology (2017) and presents the first report on the distribution and genetic diversity of GRD agents in Ghana.

The complete genome of a QLD isolate of the novel PhBMYV was determined. The genome consisted of six open reading frames (ORFs) typical of Poleroviruses, with their respective putative proteins closely related to two previously reported PhBMYV isolates from New South Wales (NSW) and Western Australia (WA), except within the RNA-dependent-RNA-polymerase (RdRp) and Coat protein-Read through (CP-RT). The RdRp only shared ~63% amino acid identity with the NSW and WA isolates and the CP-RT was distinct (33 – 34% amino acid identity with other PhBMYV isolates) and shared 53% identity with *Chickpea chlorotic stunt virus* (CpCSV). Recombination analysis using RDP4 suggested the QLD isolate was an evolutionary product of recombination between the NSW (minor parent) and WA (major parent) isolates. The virus was successfully transmitted from Phasey bean to pea (*Pisum sativum*), chickpea (*Cicer arietinum*) and to Phasey bean plants using both vector and graft transmission methods. Based on the results of this study, PhBMYV QLDCL 16 is suggested as a genetic variant of PhBMYV and perhaps represents a distinct species.

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1 CHAPTER ONE: GENERAL INTRODUCTION

1.1.1 Groundnut significance and production

Groundnut (Peanut, *Arachis hypogaea*) is a crop of global importance, widely grown in tropical and subtropical regions, by small holder and commercial producers, and is a food staple and valuable cash crop for millions of households. The seeds are a rich source of protein (22–30%), oil (44–56%), total carbohydrates (9.5–19.0%), minerals (phosphorus, calcium, magnesium and potassium) and vitamins (E, K and B group). It is ranked as the world's 13th most important food crop, 6th most important source of edible oil and 3rd most important source of vegetable protein (Waliyar *et al.*, 2007). In Ghana, groundnuts play an important dietary role where they provide high-quality cooking oil and are an important source of protein for both humans and animals. World groundnut production is approximately 42 million tonnes per year, with China, India Nigeria and the USA being the world's largest producers (FAO, 2014). Developing countries account for 97% of the global groundnut area and 94% of the global production with production concentrated in Asia and Africa (Waliyar *et al*., 2007). Sub-Saharan Africa (SSA) cultivates 40% of the world's groundnut harvested area, yet contributes only 26% of the world's groundnut production (Angelucci and Bazzucchi, 2013). This could be attributed partly to diseases of which Groundnut rosette disease is important. Ghana is ranked as the 8th largest producer of groundnuts in the world with an annual production of 0.4 million metric tonnes (USDA, 2013). The crop is grown in almost all agro-ecological zones of the country but the three northern regions (Northern, Upper East and Upper West) account for over 70% of production (Fig. 1.1) (Tsigbey *et al*., 2003). Despite the enormous potential of the crop to meet the nutritional needs of millions of people in SSA, its production has been constrained by several biotic and abiotic factors of which diseases are of significant importance.

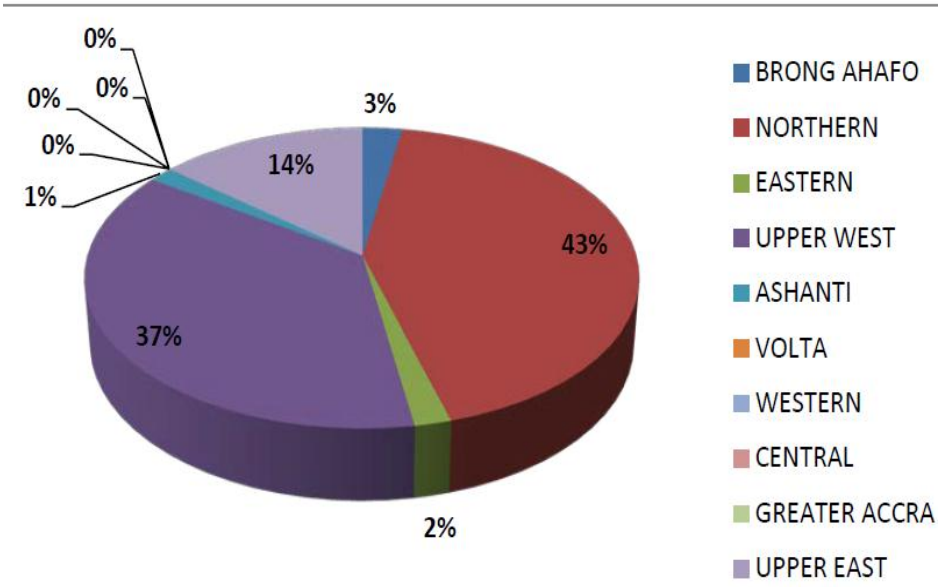

Figure 1.1 Regional distribution of groundnut production in Ghana (Angelucci and Bazzucchi, 2013)

1.2 Groundnut rosette disease

Although several diseases have been reported in groundnut in SSA, groundnut rosette disease (GRD) has been identified as the most devastating, responsible for annual yield losses of over US$150 million (Waliyar *et al.*, 2007). The disease is caused by a multi-infection complex of viral agents; *Groundnut rosette assistor virus* (GRAV): genus *Luteovirus*, *Groundnut rosette virus* (GRV): genus *Umbravirus* and its satellite RNA (SatRNA). The disease is transmitted from plant to plant by the aphid vector *Aphis craccivora* Koch (Okusanya and Watson, 1966) and induces either green rosette, chlorotic rosette (Gibbons, 1977; Murant and Kumar, 1990) or mosaic rosette symptoms in infected plants (Storey and Ryland, 1957). The major countries where the disease has been identified include Burkina Faso, Ghana, Nigeria, Malawi, Mozambique, Uganda (Olorunju *et al*., 2001), Angola, Cˆote d'Ivoire, Gambia, Kenya, Madagascar, Niger, Senegal, South Africa, Sudan, Swaziland, Tanzania and Zaire (Gibbons 1977; Naidu *et al*., 1999a). In Ghana, the disease occurs across all growing regions often resulting in total crop failure (Chapter 4 Appiah *et al*., 2016). GRD epidemics in northern Nigeria in 1975 (Yayock *et al*., 1976) and Zambia resulted in the destruction of

about 0.75 million ha and 43,000 ha of groundnut respectively while groundnut production was reduced by 23% in the central region of Malawi (Anon., 1996).

In Ghana, the average yields of 500 to 800 kg/ha are far below the potential yields of 1,800 to 2,800 kg/ha (Adu-Dapaah *et al*., 2004). This is at least partially due to GRD, which is not adequately managed and other unidentified viral diseases. In a recent study, *Groundnut ringspot virus* (GRSV) which is regarded as an emerging threat to crop production in several important crops, was identified as infecting groundnuts in Ghana (Chapter 4, Appiah *et al*., 2016). Viral diseases, besides reducing yield, may also affect the nutritive quality of the produce. The mosaic symptoms, and/or necrotic lesions induced by systemic viral infection may indicate structural changes in the chloroplasts, altered carbon metabolism and the accumulation of starch grains. Plant diseases also have the potential of altering membrane permeability in plants resulting in impaired mineral uptake (Huber and Graham, 1999).

1.3 Viral diseases of Legumes in Australia

Australia is among the world's largest exporters of legumes (pulses), including lentils (*Lens culinaris*), chickpeas (*Cicer arietinum*), faba beans (*Vicia faba* L.) and field peas (Fig. 1.2). The legume industry currently contributes about AUD$1.6 billion per year to the Australian economy (Australian Food News, 2016). Diseases caused by viruses have been identified as one of the major constraints to legume crops and pastures production worldwide (Bos *et al*., 1988; Edwardson and Christie, 1991). In Australia, several different virus species have been reported as infecting legumes (Latham and Jones, 2001a; Schwinghamer and Schilg, 2003; Thomas *et al*., 2004), the most common being *Alfalfa mosaic virus* (Bromoviridae, genus Alfamovirus) (AMV), *Cucumber mosaic virus*, (Bromoviridae, genus Cucumovirus) (CMV), *Bean yellow mosaic virus* and *Pea seedborne mosaic virus* (Potyviridae, genus Potyvirus) (BYMV and PSbMV) (Jones and Coutts, 1996). Viruses belonging to the Luteovirus family

have also been reported as infecting legumes in Australia. These include *Bean leaf roll virus* (genus Luteovirus) (BLRV), *Beet western yellows virus* (genus Polerovirus) (BWYV) (Schwinghamer *et al*., 1999; Latham and Jones, 2001a), *Soybean dwarf virus* (Wright and Jones, 2003) and more recently Phasey bean mild yellows virus (PhBMYV) (Sharman *et al*., 2016).

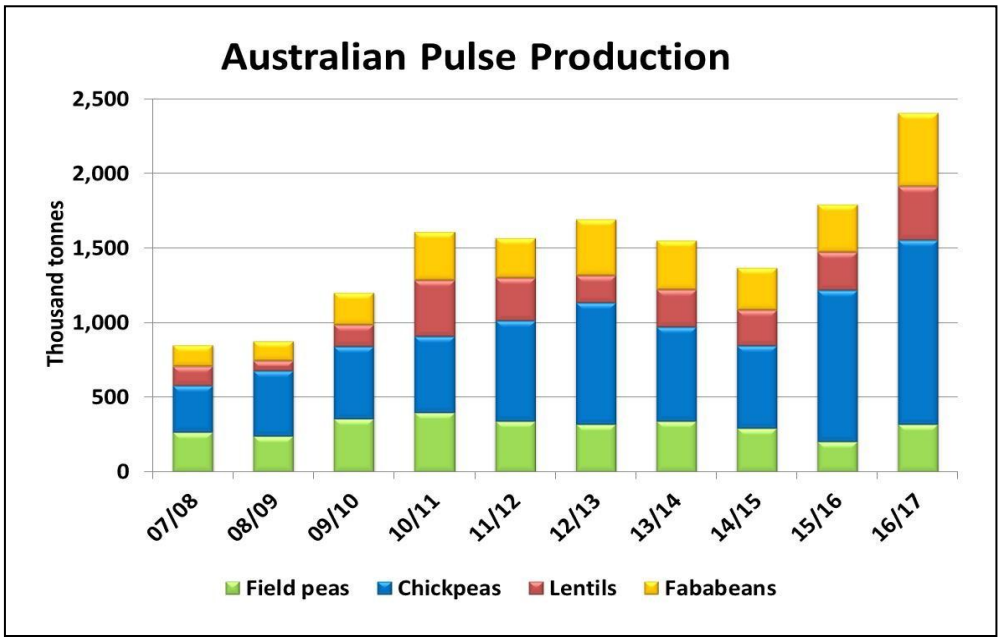

Figure 1.2 Australian pulse production (Pulse Market Insight, 2016).

1.3.1 Phasey bean mild yellows viral disease

Phasey bean mild yellows virus (PhBMYV) is a novel Polerovirus that infect legumes in Australia. The virus was first identified as infecting peas in Tasmania (Wilson *et al*., 2012) and Phasey bean in Queensland (QLD) and subsequently in Western Australia (WA) and New South Wales (NSW) as infecting *Trifolium subterraneum* (subterranean clover) and *Cicer arietinum* (chickpea) respectively (Sharman *et al*., 2016). The virus induces mild yellowing symptoms in the infected Phasey bean (Sharman *et al*., 2016) and symptomless infection in peas (Wilson *et al*., 2012). Poleroviruses have high genetic diversity and

evidence for genomic recombination is common. They are particularly associated with the emergence of new viral diseases worldwide (Lotos *et al*., 2016), and are responsible for major yield losses in vegetable and arable crops. Thus, the identification of this novel virus in Australian legumes should be of great concern. Other new Poleroviruses have also been recently reported in legumes elsewhere (Abraham *et al*., 2006; Zhou *et al*., 2012; Abraham *et al*., 2008).

1.4 Research Objectives

The low yields of groundnut usually obtained in Ghana and other countries in SSA could be partly attributed to GRD (Naidu *et al*., 1999a; Waliyar *et al.*, 2007). Cultural methods involving delaying planting to avoid the insect vectors, removal of volunteer plants, rogueing, and chemical spray have been used over the years (Naidu *et al*., 1999b) but these have not provided adequate control as the disease still persists causing severe losses. Although several rosette-resistant varieties have been released, these are only partially resistant to GRV and the satRNA and not GRAV (Olorunju *et al*., 2001). Thus more effort is needed to screen additional sources of germplasm especially in Ghana to identify more sources of resistance. Information such as virus distribution and isolate variability are of paramount importance to disease management and efficient breeding programmes. Additionally, information on the effect of the disease on the quality of groundnuts is lacking. Although several researchers have reported GRD (Adu-Dapaah et al., 2004; Olorunju *et al*., 2001; Gibbons, 1977; Naidu *et al*., 1999a) there is limited or no information on the effect of the disease on the quality of the produce.

In Australia, legumes play an important role as food for humans, feed for animals and in the export sector (Australia Food News, 2016). Farmers are increasingly incorporating pulse crops into their farming systems due to their ability to fix nitrogen and improve soil fertility.

In the year 2015, a total of 2.2 million tonnes of grain worth A$1.2 billion in exports was produced from 1.8 million ha of pulse crops. This level of production is envisaged to increase to 4.2 million tonnes with a commodity value of A$1.504 billion and a farm benefit of A$538 million assuming all constraints are overcome (Pulse Australia, 2016). The identification of a novel Polerovirus infecting diverse Australian legume crops and weeds is therefore a concern and necessitates studies which will enable informed decisions to be made regarding control strategies for the management of the disease.

This thesis will:

(i) Determine the distribution of *Groundnut rosette assistor virus* in peanut growing regions of Ghana through surveys.

(ii) Screen local germplasm for additional sources of resistance to GRD.

(iii) Determine the impact of the GRD on nutritive value of groundnuts.

(iv) Determine the genetic diversity of the three Groundnut rosette disease viral agents in Ghana.

(v) Attempt to identify the host range of the novel Phasey bean mild yellows (PhBMYV) virus.

(vi) Generate genomic sequences of the PhBMYV isolate from Queensland and Tasmania using Next Generation and Sanger sequencing techniques.

(vii) Develop virus-specific primers for its detection.

The outcome of this research is believed to serve as a guide for formulating and implementing control measures for the management of both viral diseases.

1.5 Overview of thesis content

This thesis has been prepared using the PhD thesis by publication option. As such, it is a collection of published, accepted or ready-to-be-ubmitted papers. It is important to note that

some of the content, including literature review, general introduction and methods may partially overlap between chapters.

- Chapter 1: provides a brief background, the extent of the problem, research area with specific objectives and aims of the research. It also identifies some research gaps that need attention and shows an outline of the thesis structure.

- Chapter 2: reviews important literature on Groundnut rosette disease in sub-Saharan Africa and viruses of legumes in Australia. It highlights current production levels of groundnut and pulses in SSA and Australia respectively, and identifies viral diseases as one of major constraints to production. It identifies efforts made in combating viral diseases of peanuts and research gaps that need to be addressed. It also highlights the emergence of a novel Polerovirus as a threat to legume production in Australia and the need to devise control measures.

- Chapter 3: is an experimental chapter that focusses on the impact of groundnut rosette disease on the nutritive value and elemental composition of peanuts (*Arachis hypogaea*)

  Reference: Appiah, A. S., Offei, S. K., Tegg, R. S., and Wilson, C. R. (2016). Impact of groundnut rosette disease on nutritive value and elemental composition of four varieties of peanut (*Arachis hypogaea*). *Annals of Applied Biology* 168: 400 – 408.

- Chapter 4: is an experimental chapter that focusses on screening of local Ghanaian groundnut cultivars for resistance to GRD. This chapter also reports the presence of *Groundnut ringspot virus* (GRSV) for the first time in Ghanaian groundnuts.

Reference: Appiah, A. S., Offei, S. K., Tegg, R. S., and Wilson, C. R. (2016). Varietal response to groundnut rosette disease and the first report of Groundnut ringspot virus in Ghana. *Plant Disease* 100:946-952.

- Chapter 5: is an experimental chapter focusses on assessing the genetic diversity of Groundnut rosette disease agents and the distribution of *Groundnut rosette assistor virus* in major groundnut-producing regions of Ghana

  Reference: Andrew S. Appiah, Frederick L. Sossah, Robert S. Tegg, Samuel K. Offei, Calum R. Wilson (2017). Assessing sequence diversity of Groundnut rosette disease agents and the distribution of *Groundnut rosette assistor virus* in major groundnut-producing regions of Ghana. Tropical Plant Pathology, 42:109–120.

- Chapter 6: an experimental chapter that focusses on studies on transmission, complete genome sequencing and molecular phylogeny of a novel Polerovirus from Australian legumes.

  Reference: Appiah, A.S., Tegg, R.S., Sharman, M., Wilson, C.R. Genomic analysis and transmission of Phasey bean mild yellows virus isolates from Queensland and Tasmania. Manuscript under preparation

- Chapter 7: provides a summary and conclusion for this study. It identifies gap in this research that needs further attention and provides recommendations for disease management.

2 CHAPTER TWO: LITERATURE REVIEW

2.1 Groundnut: origin, distribution, production and uses

Groundnut, also called Peanut (*Arachis hypogaea* L.) is an allo-tetraploid: 2n=40, x =10 (Ravi, 2011) and one of the world's most economically-important legumes (Dang *et al*., 2010). The crop is believed to have originated in southern Bolivia and north-western Argentina on the eastern slopes of the Andes (Rao, 1987), from where it has spread to other areas and is now cultivated in tropical and subtropical regions (Naidu *et al.*, 1999a) as well as the warmer areas of temperate regions (Hammons, 1994). The crop is grown on 26.4 million ha of land area in over 100 countries (Ntare *et al*., 2008) with more than half of the production area occurring in arid and semi-arid regions (Reddy *et al.*, 2003). The crop is cultivated on six continents between 40°N and 40°S (Naidu *et al.*, 1999a) with its geographical classification delineated into six regions namely, the Americas, Asia, Africa, New East Asia, Europe and Oceania (Gregory *et al.*, 1980).

Peanut is the world's 13th most important food crop, sixth most important source of edible oil and third most important source of vegetable protein (Waliyar *et al*., 2007). Developing countries account for 97% of the global groundnut area, contributing about 94% to the global production. Groundnut production occurs most in Asia and Africa with Asia accounting for 56% of the global area and 68% of the total global production while Africa boasts 40% of the global area and 26% of the total global production (Waliyar *et al*., 2007). On a global basis, China is the leading producer with 15.7 million metric tonnes followed by India, Nigeria and United States of America with 6.6, 3.4 and 2.4 million metric tonnes respectively (FAO, 2014). Ghana is currently ranked eighth globally and fourth in Africa with 0.44 million metric tonnes (USDA, 2013). In sub-Saharan Africa (SSA), groundnut is grown predominantly by small-land holders under rainfed conditions (Waliyar *et al.*, 2007) with the

largest producers being Nigeria, Senegal and Sudan with 1.55, 1.0 and 0.85 million metric tonnes respectively (USDA, 2013).

Although groundnut is grown in all agro-ecological zones of Ghana, the Guinea and Sudan savanna agro-ecological zones have about 85% of the total area under cultivation and account for the bulk of the nation's groundnut production (Atuahene-Amankwa *et al*., 1990). During the 2003 cropping season, 439,030 metric tonnes of groundnut were produced in Ghana from a land area of 464,710 ha with the northern sector (Northern, Upper West and Upper East regions) producing 91.4% of the national output (MOFA-SRID, 2004). In 2010, the northern sector recorded an average annual production of 498,134 metric tonnes from an average land area of 327,550 hectares (MOFA-SRID, 2011).

Peanut is a major source of protein and vegetable oil as well as providing dietary protein and vitamins (thiamine, riboflavin, and niacin) for people in many developing countries (Savage *et al*., 1994). Two thirds of the world's peanut production is processed into oil while the remaining is consumed by humans as food (Dang *et al*., 2010). The seeds of peanut contain 44-52% high quality edible oil, 26-28%, easily digestible protein and 20% carbohydrates (Waliyar *et al*., 2007), and are an important source of vitamins E, K, B1 and B3, minerals and dietary fibre (Ntare *et al*., 2008). According to Higgs (2003), peanut has high lipid content (ca. 46%) that is rich in monounsaturated fatty acids, with no cholesterol. Several researchers have shown that frequent consumption of peanuts lowers serum low density lipoprotein (LDL)-cholesterol levels, thus promoting cardiovascular health and reducing the risk of development of type II diabetes (Fraser *et al.*, 1992; Hu *et al*., 1998; Alper and Mattes, 2003). Furthermore, it has been shown to promote weight management when consumed as part of a moderate fat diet because of its satiating effect (Higgs, 2005). After harvesting, the leaves and stalk (haulms) are utilized as fodder for livestock (Marfo *et al.*, 1999) and the 'cake' that is formed after oil extraction is a rich source of protein for animal feed. The shells

are used as fuel, as filler in animal feed and in making cardboard (Waliyar *et al*., 2007). As a legume, it improves soil fertility by fixing atmospheric nitrogen (Adu-Dapaah *et al*., 2004).

In Ghana, peanut is used extensively as a source of cooking oil and in confectionery products for human consumption. Peanut hay (vine) is an important fodder resource for livestock production in northern Ghana especially, in the dry season when green forage is rarely available (Tsigbey *et al.*, 2003; Naab *et al*., 2005). Despite its numerous benefits, the production of the crop has been constrained by several diseases of which viral diseases are of economic importance.

2.2 Diseases and Pests of groundnut

Diseases and pests are a major constraint to the production of groundnut throughout the world. Several diseases caused by bacteria, fungi, nematodes, parasitic flowering plants, viruses and mycoplasmas have been identified in groundnuts (Subrahmanyam *et al*., 1992) and are responsible for low yields (McDonald *et al*., 1998). Bacterial wilt of groundnut, caused by infection with *Pseudomonas solanacearum* is the only important bacterial disease of groundnut, occurring in groundnut-producing areas of Africa and Asia (Mehan *et al*., 1985). Fungi cause seed rots and seedling diseases such as root rot, stem rot, wilts, blight, pod rot and foliar diseases. Early and late spot diseases caused by *Cercospora arachidicola* Hori and *Cercosporidium personatum* (Berk and Curt.) respectively have been identified as the most important foliar diseases of groundnut in the world (Ogwulumba *et al*., 2008; Smith, 1984). Rust (*Puccinia arachidis* Speg.) has also been found to infect groundnut worldwide causing serious losses (Subrahrnanyam *et a1*., 1985). Several viral diseases are known to be constraints to production in all groundnut-growing areas of the world (Reddy, 1988). In SSA, GRD has been found to be the most devastating (Waliyar *et al*., 2007).

Globally, the most important insect pests found on groundnut are aphids (*Aphis craccivora*), thrips (*Frankliniella spp*.), jassids (*Empoasca dolichi*), white grubs (larvae of various beetles), termites (mainly *Microtermes spp*.), millipedes, ants and the red tea bug *Hilda patruelis*. Aphids are particularly harmful because they are vectors of GRD. Root-knot nematodes including *Meloidogyne arenaria, Meloidogyne hapla* and *Meloidogyne javanica* may also cause considerable yield loss in groundnut (Starr *et al*., 2002).

Storage pests include Bruchid beetles (*Caryedon serratus, Callosobruchus spp*., *and Acanthoscelides spp*.) and flour beetles (*Tribolium spp*.). Parasitic plants (*Alectra vogelii* Benth. and *Striga spp*.) have also been reported as causing damage to groundnut in various African countries (Ntare, 2007).

2.2.1 Groundnut rosette

Groundnut rosette has been reported as the most destructive virus disease of groundnut (*Arachis hypogaea* L.) in sub-Saharan Africa (Reddy, 1991) occurring wherever the crop is grown. The disease was first reported in 1907 from Tanganyika, now called Tanzania (Zimmermann, 1907). It is restricted to the African continent and its off-shore islands, including Madagascar (Naidu *et al*., 1999a) (Fig. 2.1), and is responsible for devastating losses to groundnut production (Naidu *et al*., 1998). The major areas of disease occurrence include Burkina Faso, Ghana, Nigeria, Malawi, Mozambique and Uganda (Olorunju *et al*., 2001). The disease has also been reported in Angola, Côte d'Ivoire, Gambia, Kenya, Madagascar, Niger, Senegal, South Africa, Sudan, Swaziland, Tanzania and Zaire (Gibbons, 1977; Naidu *et al*., 1999a).

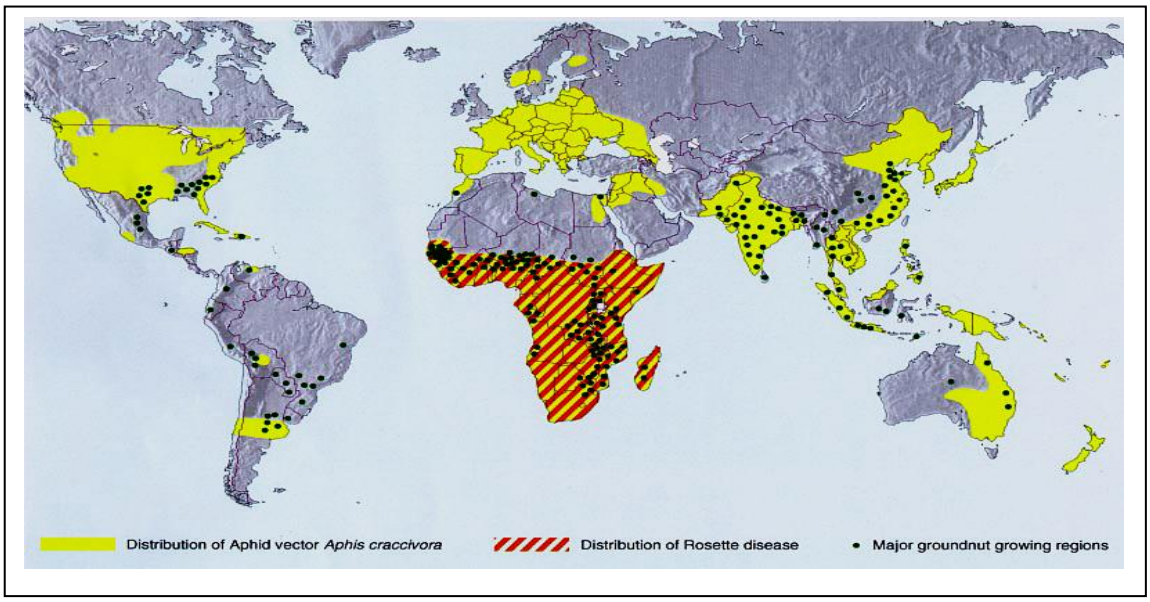

Figure 2.1 Worldwide distribution of groundnut, the groundnut rosette disease and its aphid vector (Naidu *et al*., 1999b).

According to Subrahmanyam *et al*. (1997) GRD outbreaks are sporadic and unpredictable, but can result in yield losses of up to 100%. According to Yayock (1976), an epidemic of GRD in northern Nigeria in 1975 resulted in the destruction of approximately 0.75 million hectares of groundnut, with an estimated loss of about US$250 million in regional trade. A similar epidemic in eastern Zambia in 1995 affected approximately 43, 000 ha of groundnut, with an estimated loss of US$4.89 million. In 1996, groundnut production was reduced by 23% in the central region of Malawi as a result of groundnut rosette (Anon., 1996).

Losses in yield of groundnut GRD depend largely on the plant growth stage at which infection occurs. According to Naidu *et al*. (1999b), a 100% loss in pod yield may result if infection occurs before flowering with variable losses when infection occurs between flowering and pod maturing stage. After this stage, any infections normally cause negligible effects.

2.2.1.1 Etiology and vector of GRD

Groundnut rosette is caused by infection with a complex of two viruses; *Groundnut rosette virus* (GRV) genus *Umbravirus* (Murant *et al.,* 1995), and its satellite ribonucleic acids (satRNA) (Blok *et al*., 1995), and the *Groundnut rosette assistor virus* (GRAV) genus *Luteovirus* (Reddy *et al*., 1985**).** GRV does not encode a coat protein, and its genomic RNA is encapsidated in the coat protein of GRAV **(**Casper *et al*., 1983). The satGRV is essential for this encapsidation and symptom development by GRV. The GRV in turn potentiates the replication of satGRV (Fig. 2.3). On their own, infection with either GRAV or GRV results in symptomless or transient mild mottle symptoms. Murant *et al*. (1988) showed that GRV cultures lacking satellite RNA induce no symptoms, or only transient chlorotic leaf mottling in groundnut, suggesting that the satellite RNA is essential for the rosette symptom induction. According to Murant and Kumar (1990), variants of the satellite RNA are responsible for the different forms of symptoms; chlorotic rosette (Fig. 2.2) and green rosette as reported by Waliyar *et al*. (2007) and Gibbons (1977). Findings by Murant (1990) revealed that aphid transmission of GRV depends not only on GRAV but also on the satGRV, explaining why satellite-free isolates of GRV have not been found in nature.

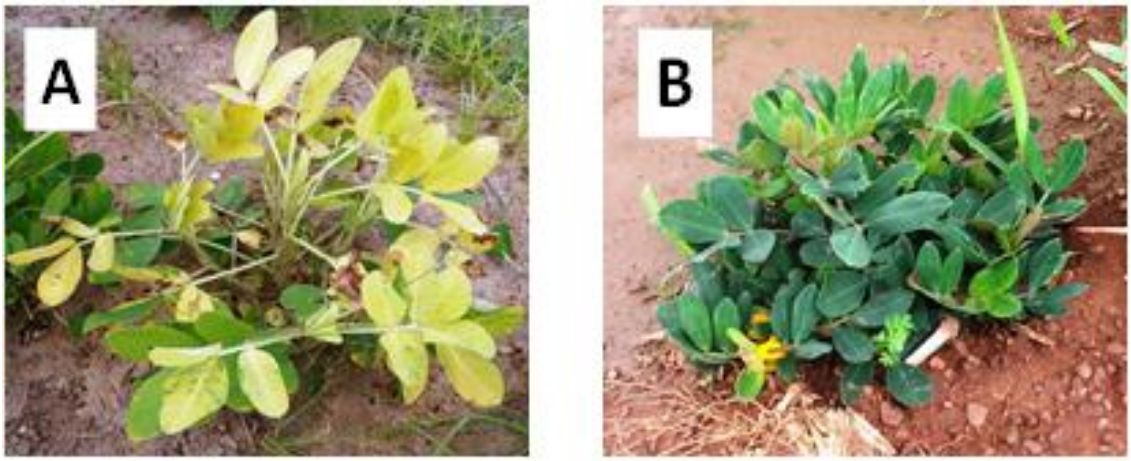

Figure 2.2 Peanut plants showing typical symptoms of GRD **A**. chlorotic rosette and **B**. green rosette

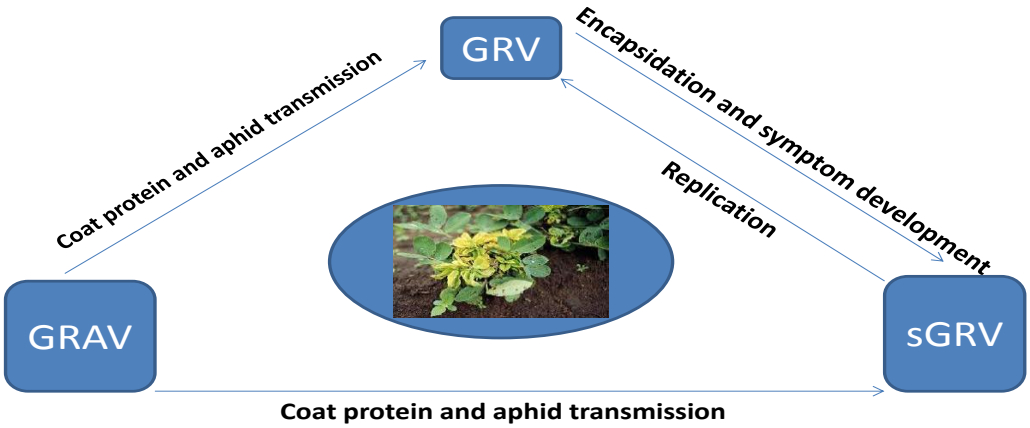

Figure 2.3 Interaction of GRV, GRAV and sGRV in groundnut rosette disease development and vector transmission.

Groundnut rosette is transmitted by the cowpea aphid (*Aphis craccivora* Koch) in a persistent, circulative manner (Okusanya and Watson, 1966). Even though a single aphid vector acquires all three disease agents during feeding on infected plants, it can transmit either GRAV or GRV and its satellite separately (Naidu and Kimmins, 2007). This implies that symptomatic plants do not necessarily contain the aphid-transmissible GRAV. According to Misari *et al*. (1988a), *A. cracivora* requires 4 hrs and 8 hrs to acquire GRV from chlorotic and green rosette plants respectively. The latent period varied from 1 to 11 days with median latent periods of 26.4 hr and 38.4 hr respectively for chlorotic and green rosette. Inoculation access period for aphids fed on both symptom forms were 10 min after 24 hr latency. The maximum retention time was found to be the lifetime of the aphids which was approximately 14 days. On the contrary, Marion *et al*. (1967) revealed that most *A. craccivora* required longer than 24 h acquisition feeding on infected groundnuts to acquire the virus, and many needed inoculation access period of 2-3 days to cause infection, even after several days of feeding on infected plants. The delay partly reflects the slow uptake of virus and possibly a period

needed for virus multiplication in aphid tissue but some is lost through resistance of the test plants to infection.

2.2.1.2 The Groundnut rosette assistor virus (GRAV)

GRAV was first recognised and named by Hull and Adams (1968) as one of the agents of Groundnut rosette, acting as a helper virus for aphid transmission of GRV and sat RNA (Murant, 1990). It was identified as a member of the *Luteoviridae* (Casper *et al*., 1983; Reddy *et al*., 1985) and found to be serologically related to several *Luteoviruses,* reacting with polyclonal antisera raised against *Bean leafroll virus* (BLRV), *Beet western yellows virus* (BWYV) and *Potato leafroll virus* (PLRV) in immunosorbent electron microscopy tests, and with antiserum to BWYV in ELISA. Furthermore, Rajeshwari *et al.* (1987) found that three out of ten anti-PLRV monoclonal antibodies (MAbs) raised by Massalski and Harrison in 1987 reacted with GRAV particles in triple-antibody sandwich ELISA using PLRV or BWYV polyclonal antisera as capture antibodies.

The virus is transmitted by *A. craccivora* and the melon or cotton aphid; *A. gossypii* in a persistent manner. It is retained when the vector moults, does not replicate in the vector and is not transmitted congenitally to the progeny of the vector (ICTVdB Management, 2006).

GRAV alone causes no obvious symptoms in infected groundnut plants, although it may be responsible for minor yield reductions (Scott *et al.*, 1996). Because GRV RNA and sat RNA are encapsidated within the CP of GRAV, the vector transmission characteristics of groundnut rosette are influenced by GRAV and not the other two agents. Therefore, GRAV plays a significant role in the epidemiology and perpetuation of groundnut rosette with infected plants lacking GRAV regarded as ‘terminal’ for the spread of the disease (Naidu and Kimmins, 2007).

Groundnut is the only known natural host of GRAV, but experimentally the virus has been found to infect seven other species within the family *Leguminoceae (Pisum sativum, Stylosanthes gracilis, S. hamata, S. mucronata, S. sundaica, Trifolium incarnatum* and *T. pratense)* and four species from other families *(Capsella bursa-pastoris, Gomphrena globosa, Montia perfoliata* and *Spinacia oleracea)* (Okusanya and Watson, 1966; Rajeshwari and Murrant, 1988; Hull and Adams, 1968). In all of these hosts, virus infection resulted in no obvious symptoms except in *C. bursa-pastoris,* in which chlorosis may occur.

The genome of GRAV consists of a linear, single-stranded RNA, 6.9 kb in size. Replication of GRAV occurs in the cytoplasm and does not depend on a helper virus (Waliyar *et al*., 2007).

2.2.1.3 Groundnut rosette virus

Groundnut rosette virus (GRV) is a self-replicating ssRNA which does not produce a coat protein and therefore has no conventional particles. Virions are associated with its helper virus (GRAV) and are dependent on co-infection of this helper virus during replication. It requires GRAV for its transmission, and the satellite RNA for symptom induction. GRV is required for the replication of the satellite RNA (2.7kb) which is also encapsidated within the GRAV virion. GRV is transmitted by the aphid *A. cracivora* in a persistent manner when co-infected by GRAV. The virus is retained when the vector moults, does not multiply in the vector, and is not transmitted congenitally to the progeny of the vector. It is transmitted by mechanical inoculation and grafting but not by contact between plants or by seed (Brunt *et al*., 1996).

Eleven plant species, including *A. hypogaea*, have been shown to be susceptible hosts of GRV within families *Chenopodiaceae, Leguminosae-Papilionoideae* and *Solanaceae* under experimental conditions. These are *Arachis hypogaea*, *Chenopodium amaranticolor*, *C.*

murale, *C. quinoa*, *Glycine max*, *Nicotiana benthamiana*, *N. clevelandii*, *N. rustica*, *Nicotiana x edwardsonii*, *Trifolium incarnatum*, *T. repens*. The most suitable diagnostic plants are *A. hypogaea*, *N. clevelandii*, expressing necrotic rings, systemic curling and malformation and *Chenopodium amaranticolor*, showing chlorotic local lesions (Brunt *et al*., 1996).

The single-stranded RNA of GRV comprises 4019 nucleotides and contains four large open reading frames (ORFs) (Fig. 2.4). The second ORF from the 5' end contains sequences that encode motifs characteristic of a viral RNA-dependent RNA polymerase and is probably expressed by a -1 frameshift mechanism as a fusion protein with the product of the 5'-most

ORF. The other two ORFs that are probably expressed from subgenomic RNAs, are almost completely overlapping in different reading frames. One of the putative products shares sequence similarity with viral movement proteins. None of the proteins encoded by GRV RNA are structural proteins (Taliansky *et al*., 1996)

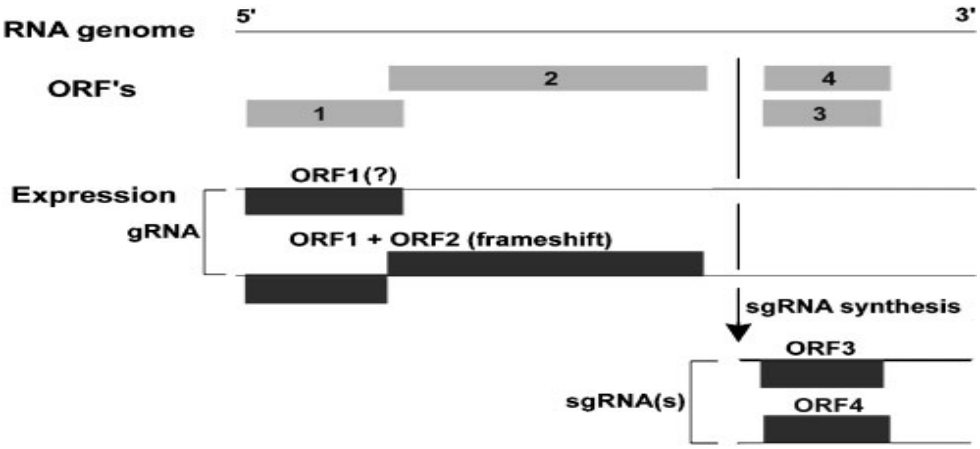

Figure 2.4 The genome of GRV showing the different expression strategies: production of subgenomic RNA(s) (sgRNA(s), initiation of translation at the first optimal AUG on the genomic RNA (gRNA; ORF1) or sgRNA(s) (ORF3 and ORF4)), and initiation of translation as for ORF1 and frameshift (ORF1+ORF2). Lines represent RNA molecules, grey boxes

represent open reading frames and black boxes represent translation products (Taliansky *et al*., 2003a).

2.2.1.4 Satellite RNA of GRV

The GRV-satRNA is 895 to 903 nt long (Murant, 1990) and its variants have been shown to be responsible for the different forms of symptoms in GRD (Murant and Kumar, 1990). It has also been established that aphid transmission of GRV depends not only on GRAV, but also on the GRV satellite RNA (Murrant, 1990). Although no virus-like particles have been seen in plants infected with GRV alone, such plants have been found to contain abundant dsRNA forming a characteristic electrophoretic band pattern with three major species; 4.6 kbp (dsRNA-I), 1.3 kbp (dsRNA-2) and 900 bp (dsRNA-3) (Murant *et al*., 1988). Preparations containing the dsRNA species become infective only when heat-denatured, indicating that the infective RNA molecules are single-stranded. It has been shown that dsRNA-3 is a double-stranded form of a satellite RNA which depends on RNA-1 for replication in plants while dsRNA-2 seems to represent a double-stranded form of a sub-genomic fragment of RNA-1 (Murant *et al.,* 1988).

2.2.1.5 Role of Satellite RNAs in symptom expression by plant viruses

Viral satellites are viruses or nucleic acids that depend on a helper virus for replication but are not essential for the replication of the helper virus and lack appreciable sequence homology with the helper virus genome (Murant and Mayo, 1982). Unlike satellite viruses, SatRNAs do not encode their own coat proteins. They are either separately encapsidated within the coat protein of their helper viruses, or in association with the viral RNA(s)

depending on the particular satRNA (Collmer and Howell, 1992). Satellite RNAs are relatively short molecules, usually <1,500 nt.

A unique feature of satellite RNAs that interests plant virologists is the ability to alter symptoms produced by plant viruses. While most viral satellites such as the satellites of *Tobacco ringspot virus* (TobRV) attenuate disease, others may exacerbate symptoms of the disease produced by the virus alone or the virus in association with another, avirulent satellite (Li and Simon, 1990). Furthermore, some satellites produce new symptoms that are not associated with the helper virus alone, examples being *Lethal tomato necrosis*, *Brilliant yellowing of tobacco*, and chlorosis of tomato caused by satRNAs of *Cucumber mosaic virus* (CMV), hop nettlehead caused by a satRNA of *Arabis mosaic virus* (ArMV); and Groundnut rosette caused by the satellite-like RNAs of GRV (Collmer and Howell, 1992). According to Kumar *et al*. (1991) some Malawian cultures of GRV induced brilliant yellow blotch mosaic symptoms in *N. benthamiana*, instead of the characteristic veinal chlorosis and mild mottle. Nevertheless, the usual chlorotic type of rosette symptoms in groundnut was still evident.

2.3 Other viruses of Groundnut

About 31 viruses have been reported to naturally infect groundnut worldwide. Those of global or regional economic importance include *Tomato spotted wilt virus* (TSWV), *Groundnut bud necrosis virus* (GBNV), *Tobacco streak virus* (TSV), *Peanut clump virus* (PCV), *Peanut stripe virus* (PStV), a strain of *Bean common mosaic virus* (BCMV), *Peanut mottle virus* (PeMoV) and CMV, GRV and GRAV (Sreenivasulu *et al*., 2008). In addition to GRAV and GRV (Naidu *et al*., 1999a), the following viruses have been reported as naturally infecting groundnuts in West Africa: *Cowpea mild mottle virus* (CPMMV) (Iizuka *et al*., 1984), Groundnut chlorotic spotting virus (GCSV) (Fauquet *et al*., 1985; Dollet *et al*., 1987), Groundnut eyespot virus (GEV) (Dubern and Dollet, 1980), Peanut clump virus (PCV)

(Thouvenel *et al*., 1976), Peanut yellow mottle virus (PeYMV) (Lana, 1980), TSWV (Culbreath *et al*., 2003) and more recently GRSV (Appiah *et al*., 2016). GRSV belongs to the genus Tospovirus in the family Bunyaviridae, is not seed borne, and is naturally vectored by several species of thrips from the genus *Franklinella* (Pappu *et al*., 2009). GRSV is regarded as an emerging threat to crop production in several crops of economic importance, including groundnut. The virus was first identified in groundnuts from South Africa (de A´ vila *et al*., 1993) and subsequently in Argentina (de Breuil *et al*., 2007), Brazil (Camelo-Garc´ıa *et al*., 2014) and Ghana (Appiah *et al*., 2016). The virus has also been found as infecting other crops such as tomato (Webster *et al*., 2010), cubiu (Boari *et al*., 2002), cucumber (Spadotti *et al*., 2014) and watermelon (Leão *et al*., 2014).

2.4 Host resistance to GRD

Sources of resistance to GRD in groundnut were first discovered in 1952 in landraces of the late-maturing Virginia (*A. hypogaea* L. subsp. *hypogaea* var. *hypogaea*) from Burkina Faso and Cote d.Ivoire (Catherinet *et al*., 1954) and has since formed the basis for breeding programmes throughout Africa (Olorunju *et al*., 2001). The resistance was found to be controlled by two recessive genes and was effective against the GRV and its sat RNA (Bock *et al*., 1990) and might not be inherited (Misari *et al*., 1988b). Furthermore, GRV resistant lines are not immune to the virus and individual plants can succumb to the disease under heavy inoculum pressure (Wynne *et al*., 1991). Resistance to GRAV on the other hand, has not been identified (Chiyembekeza *et al*., 1997) and all rosette-resistant lines and genotypes are susceptible to the virus (Subrahmanyam *et al*., 1998). Evaluation of groundnut germplasm resulted in the identification of several GRV-resistant sources (Subrahmanyam *et al*., 1998; Olorunju *et al*., 2001). Resistance to the aphid vector has been identified in some groundnut breeding lines and is controlled by a single recessive gene **(**Herselman *et al*., 2004). However,

aphid-resistant sources have been shown to be susceptible to GRAV and GRV as well as the sat RNA (Minja *et al*., 1999).

2.5 Current management strategies for GRD and the way forward

The devastating nature of GRD necessitates the use of several methods by small holder farmers to manage the disease. Removal of volunteer groundnut plants that serve as sources of inoculum to perpetuate the disease, rogueing, cultural practices that disrupt vector movement, the control of aphid vectors with insecticides and use of rosette disease resistant cultivars are among the control strategies being used currently (Naidu *et al*., 1999b). GRD is a polycyclic disease and each infected plant serves as a source of inoculum for disease spread in the field (Naidu *et al*., 1998). Thus, the removal of volunteer groundnut plants helps in the control of the disease by either eliminating or reducing sources of inoculum.

The long feeding period required by the vector to acquire the virus provides an opportunity for their control with insecticide sprays before they spread the disease to healthy plants in the field. However, factors such as timing of spray, dosage and the type of insecticide are critical in achieving the desired results. According to Davies (1975), Menazon, used at a rate of 294 g a.i./ha and applied four times at ten day intervals starting ten days after plant emergence, was effective in reducing groundnut rosette incidence. The high cost and unavailability of insecticides however, make their use unattractive to farmers (Waliyar *et al.*, 2007). Moreover, regular insecticide application increases the risk of development of aphid resistance and poses risks to the environment and human health.

Early sowing in the rainy season to take advantage of the low aphid population and the removal and destruction of early infected plants may also help to reduce the source of inoculum and help curtail the spread of the disease (Waliyar *et al.*, 2007). However, this may

not be very effective since plants normally become infected before becoming symptomatic and removed; and virus spread may occur from the asymptomatic plants. Intercropping peanut with fast growing cereals such as maize, sorghum, pearl millet and cowpea have been shown to interfere with the movement and colonization of the aphid vectors hence reducing GRD incidence (Alegbejo, 1997). Identification and destruction of alternative hosts would also help to control the disease by breaking the disease cycle.

The use of multiple control measures in an integrated manner may help to minimise the impact of the disease and boost production. According to (Waliyar *et al.*, 2007), there is a need to develop models to forecast epidemics in order to prevent future occurrence of GRD epidemics. This may however, require data on risk factors such as aphid population dynamics, inoculum source and its abundance, cropping patterns and weather parameters that favour vectors so as to maximize the effectiveness of various GRD management strategies .

Control of the disease has been difficult despite the availability of numerous control measures. Resistant varieties mostly succumb to the virus either due to excessive inoculum build-up or by a more virulent strain. Breeding for robust resistance to virus infection is the most effective control strategy available for managing the disease. A prerequisite for a more efficient breeding programme for virus resistance is to identify virus distribution and characterise viral isolates.

2.6 Family Luteoviridae

Luteoviridae is a family of single-stranded RNA plant viruses. The name is derived from a Latin word 'luteus' meaning yellow, since all original members of the group are known to induce yellowing or chlorotic symptoms in their hosts (Martin *et al.*, 1990). The taxonomy within the family is determined by the arrangement of the genome. According to ICTVdB (2013), there are 28 established members, 15 of which are classified under the three main genera; *Luteovirus* (six species), *Polerovirus* (13 species) and *Enamovirus* (one species) (Table 2.1). The other eight species are currently unassigned to any genus within the *Luteoviridae* since their genomes have not been fully characterized. Nevertheless, they resemble luteovirids in their biological properties and coat-protein (CP) sequences.

Table 2.1 Established members of Family Luteoviridae (ICTV, 2013)

Nr	**Species Name**	**Abbreviation**
Genus Luteovirus		
1	*Barley yellow dwarf virus- MAV*	BYDV-MAV
2	*Barley yellow dwarf virus-PAV*	BYDV-PAV
3	*Barley yellow dwarf virus-PAS*	BYDV-PAS
4	*Bean leafroll virus*	BLRV
5	*Rose spring dwarf-associated virus*	RSDaV
6	*Soybean dwarf virus*	SbDV
Genus Polerovirus		
1	*Beet chlorosis virus*	BChV
2	*Beet mild yellowing virus*	BMYV
3	*Beet western yellows virus*	BWYV
4	*Carrot red leaf virus*	CtRLV
5	*Cereal yellow dwarf virus-RPS*	CYDV-RPS
6	*Cereal yellow dwarf virus-RPV*	CYDV-RPV
7	*Chickpea chlorotic stunt virus*	CpCSV

8	*Cucurbit aphid-borne yellow virus*	CABYV
9	*Melon aphid-borne yellows virus*	MABYV
10	*Potato leafroll virus*	PLRV
11	*Sugarcane yellow leaf virus*	ScYLV
12	*Tobacco vein distorting virus*	TVDV
13	*Turnip yellows virus*	TuYV
Genus Enamovirus		
1	*Pea enation mosaic virus – 1*	PEMV-1
Unassigned Luteoviridae		
1	*Barley yellow dwarf virus-GPV*	BYDV-GPV
2	*Barley yellow dwarf virus-RMV*	BYDV-RMV
3	*Barley yellow dwarf virus-SGV*	BYDV-SGV
4	*Chickpea stunt disease associated virus*	CpSDaV
5	*Groundnut rosette assistor virus*	GRAV
6	*Indonesian soybean dwarf virus*	ISDV
7	*Sweet potato leaf speckling virus*	SPLSV
8	*Tobacco necrotic dwarf virus*	TNDV

http://ictvonline.org/virusTaxonomy.asp?taxnode_id=20111141

Additional members of *Luteoviridae* have been discovered and characterised but as yet formally remain unclassified (Chomič, 2011). These proposed new *Luteoviruses* include Barley yellow dwarf virus-ORV (oat redleaf virus); BYDV-ORV (Robertson and French, 2007), Chickpea yellows virus; CpYV (Abraham *et al*., 2008), Cotton leafroll dwarf virus; CLRDV (Corrêa *et al*., 2005), Lentil stunt virus; LStV (Abraham *et al*., 2008), Pepper vein yellows virus; PeVYV (Murakami *et al*., 2011), Pepper yellow leaf curl virus; PYLCV (Dombrovsky *et al*., 2010), Pepper yellows virus; PYV (NCBI* Nr FN600344), Suakwa aphid-borne yellows virus; SABYV (Shang *et al*., 2009) and Wheat yellow dwarf virus-GPV; WYDV-GPV (Zhang *et al*., 2009). Apart from the BYDV-ORV (*Luteovirus*), all the others belong to the genus *Polerovirus*.

2.6.1 Host range Luteoviruses

Most *Luteoviruses* have a limited host range (e.g. *Potato leafroll virus*, PLRV; *carrot redleaf virus*, CRLV) whereas a few like *Beet western yellows virus* (BWYV) have a very broad host range (Martin *et al*., 1990). Members of *Luteoviridae* are of serious economic concern to global agriculture since their preferred hosts include the world's most important agricultural crops. These include cereals (barley, oat, wheat, maize, rice, etc.), legumes (chickpea, faba bean, groundnut, soybean, pea, etc.), potato (and sweet potato), various cucurbit crops (watermelon, melon, squash, zucchini, etc.), sugarcane and sugar beet. These crops are the main source of proteins and carbohydrates to billions of people worldwide. According to Stevens *et al*. (2008), *Turnip yellows virus* (TuYV) was responsible for losses worth 60-90 million pounds in oil seed rape production in the UK. In Australia and New Zealand alone, an average annual loss of NZ$ 70m has been reported for *Barley yellow dwarf viruses* (Johnstone, 1995).

2.6.2 Vector Association and Transmission

Luteoviruses are transmitted in a persistent, circulative and non-propagative manner by their aphid vectors (Duffus, 1977), but not congenitally to the progeny of the vector and are retained when the insect moults (Eskandari *et al*., 1979). Each Luteovirus is transmitted by a limited number of aphid species, indicating a high level of vector-specificity. They are generally phloem-limited in their hosts and aphids acquire them for transmission while feeding on phloem sieve tube elements of host plants. Acquisition by aphids requires specific virus recognition at, and transport through the epithelial tissues of the aphid gut before release into the hemocoel. The virus is transported through the insect hemocoel, possibly associated with a chaperone-like protein and enters the insect's salivary glands again following specific recognition at the organ membrane. Transmission may then occur with the

virus moving from the salivary glands into the common salivary duct that extends the length of the stylets (Gray and Gildow, 2003). Acquisition and inoculation access feeding times by aphids have been reported to range from 0.1-4.0 h and 0.2-1.0 h respectively (Thottappilly *et al.*, 1977). On the contrary, Elnagar and Murant (1978) observed that most *Luteoviruses* require 24 hrs of feeding access time for both acquisition and inoculation to achieve efficient transmission with the minimum latent period between 12 and 24 hrs. According to Gray and Wildow (2003), this minimal latent period may extend to as much as 3–4 days. Median latent periods are 35-50 h at 15-20°C for BYDV-RPV, 44-65 h at 15-20°C for BYDV-MAV (Van der Broek and Gill, 1980) and 30-49 h at 20-25°C for PLRV (Eskandari *et al*., 1979). The length of the latent period is influenced by many factors such as the concentration of virus in the plant tissue, inherent transmission efficiency of each aphid population for a specific virus isolate as well as environmental factors such as temperature (Power and Gray, 1995; Van der Broek and Gill, 1980).

Members of the *Luteoviridae* are not transmitted by contact between hosts, through the seeds nor by pollen and mechanical sap inoculation (Brunt *et al*., 1996). However, a recent study by Mayo *et al*. (2000) has shown that co-infection of *Nicotiana benthamiana* with GRV and PLRV or BMYV (genus *Polerovirus*) resulted in the polerovirus becoming mechanically transmissible. Furthermore, PLRV was transmissible from mixtures with the CMV (ORF4) recombinant, in which the CMV movement protein gene had been replaced with that of GRV (Ryabov *et al*., 2001). Luteoviruses can also be transmitted by grafting.

2.7 Genome Organization of Luteoviruses

All members of the *Luteoviridae* share common genomic structural and organizational features. Their genomes are positive-sense ssRNA of about 1.8-2 x 106 Mr (5.5-6.0 kb) with a genome-linked protein (VPg) covalently attached at the 5' end of the RNA molecule (Mayo

et al., 1982; Murphy *et al.*, 1989) which is not polyadenylated at the 3'-end (Mayo *et al.*, 1982; Murphy *et al.*, 1987). Furthermore, the 3' ends do not appear to possess tRNA-like structures such as those associated with other plant viral groups (Miller *et al*., 1988). Comparison of the BYDV-PAV, PLRV, and BWYV nucleotide sequences suggests the existence of at least two major Luteovirus genome organizations (Martin *et al*., 1990) which separate the *Luteoviruses* (e.g. BYDV-PAV) and *Polerovirus* (e.g. PLRV).

2.7.1 The Polerovirus Genome

The genome is monopartite, linear, positive sense ssRNA (ssRNA(+)) of approximately 6 kb with a VPg bound at the 5' end with no poly(A) tail or tRNA-like structure at the 3' end. The virion RNA is infectious, serving as both genome and viral messenger RNA. The genome codes for six open reading frames (ORFs) designated ORF0, ORF1, ORF2, ORF3, ORF4 and ORF5 (Domier, 2012). When the genomes of the three virus genera in the family *Luteoviridae* are compared, the *Poleroviruses* can be distinguished from *Luteoviruses* by the presence of ORF 0, while the absence of ORF4 differentiates *Enamoviruses* from *Poleroviruses* (Domier, 2012) (Fig. 2.5) ORF0 is believed to be a suppressor of post-transcriptional gene silencing (Pfeffer *et al*., 2002). ORFs 1 and 2 encode the replication-related proteins through a ribosomal frameshift near the end of ORF1 (D'Arcy *et al*., 2000). In Polero- and *Enamoviruses*, ORF 1 overlaps ORF 2 by more than 400 nt while in *Luteoviruses* it overlaps ORF 2 by less than 20 nt. The intergenic region between ORFs 2 and 3 is about 200 nt in Polero- and *Enamoviruses* and 100 nt in *Luteoviruses* (Domier *et al*., 2002). ORFs 3 and 5 encode the coat and readthrough proteins of the viruses (Domier *et al*., 2002).

PLRV sgRNA2 may code for two viral proteins of 7.1 kDa (ORF6) and 14 kDa (ORF7) with the latter displaying nucleic acid binding activity. In CABYV, the proteins are of 8.7 and 8.3

kDa in size for ORF6 and ORF7 respectively (Ashoub *et al*., 1998). Comparisons of the nucleotide and predicted amino acid sequences by Miller *et al*. (1995) of luteoviruses with other members of the family and with different virus families identified RNA recombination as an important factor in the generation of new species within the *Luteoviridae.*

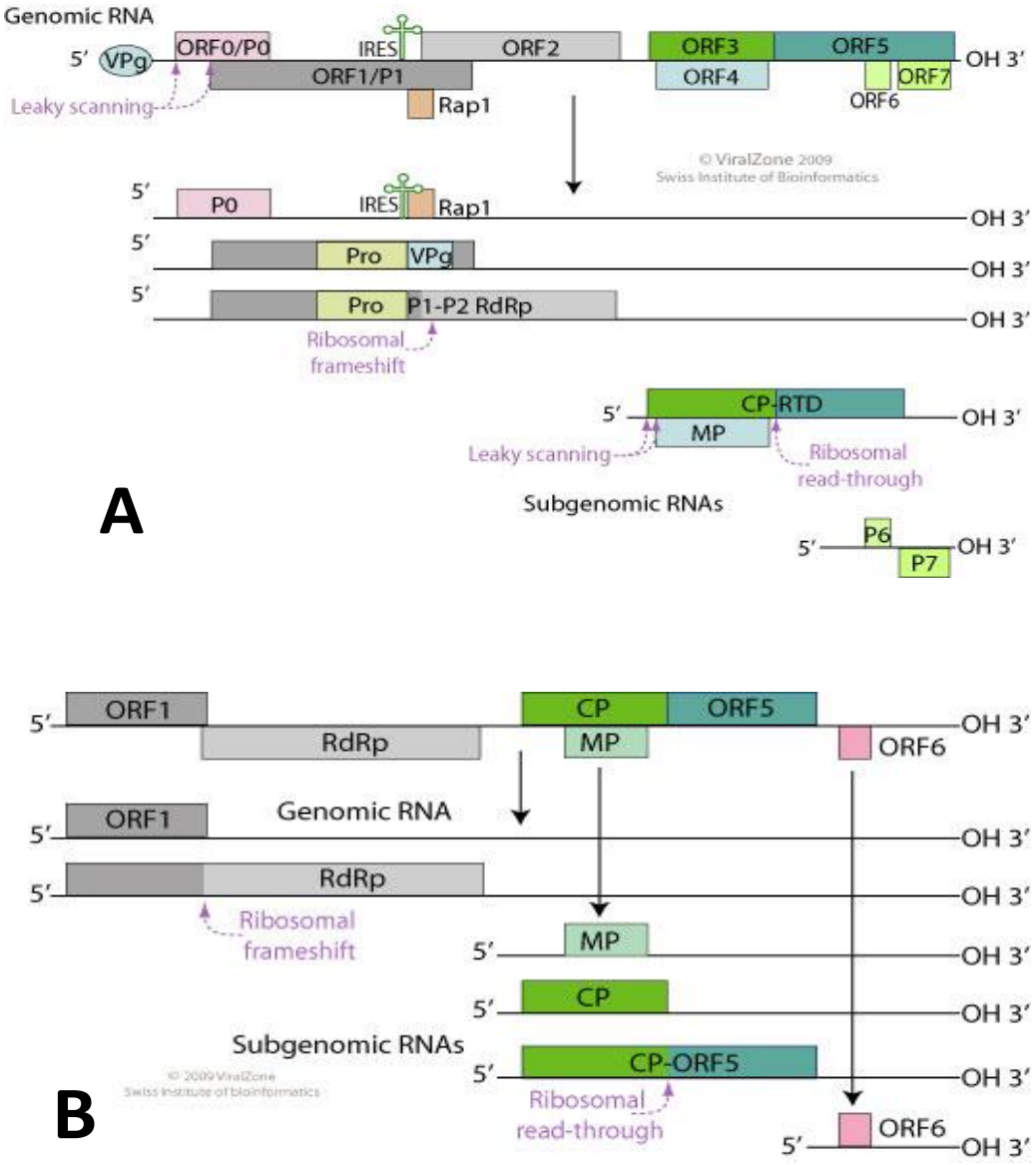

Figure 2.5 Genomes of family Luteoviridae compared A. PLRV (genus Polerovirus) and B. BYDV-PAV (genus Luteovirus) (Viral Zone, Swiss Institute of Bioinformatics).

2.7.2 Poleroviruses of legumes

Several cool- and warm-season pulse crops (grain legumes) including chickpea, field pea, faba bean, lentil, cowpea and pigeon pea are grown in rotation with cereals and pasture, forming sustainable farming systems in Australia (Siddique and Sykes, 1997). Australia's legume industry currently contributes AUD$1.6 billion per year to the Australian economy, making the country one of the world's largest exporters of legumes (Australian Food News, 2016). Virus diseases present major biotic constraints to the production of legumes, especially in the tropical and subtropical regions (Sastry and Zitter, 2014). At least 150 viruses belonging to different genera are known to naturally infect cultivated food legumes worldwide (ICTV, 2012) and several different virus species have been identified as infecting legumes in Australia (Latham and Jones, 2001a; Schwinghamer and Schilg, 2003; Thomas *et al*., 2004).

Two important viruses belonging to the family Luteoviridae namely *Bean leaf roll virus* (genus Luteovirus) (BLRV) and *Beet western yellows virus* (genus Polerovirus) (BWYV) (Schwinghamer *et al*., 1999; Latham and Jones, 2001a; Freeman and Aftab, 2001; Van Leur *et al*., 2003; Aftab *et al*., 2005) have been found in pulse crops in Australia. Members of this family are considered to some of the most destructive viral diseases of crops worldwide (Bos *et al*., 1988). Of the three virus genera of family *Luteoviridae*, members of the *Polerovirus* genus are emerging as a threat to food production worldwide (Lotos *et al*., 2016). New Poleroviruses have been identified as infecting crops including cotton in Australia and Argentina (Ellis *et al*., 2013; Distefano *et al*., 2010), strawberry in Canada (Xiang *et al*., 2015), cabbage, maize and brassicas in China (Zhang *et al*., 2014; Wang *et al*., 2016; Xiang *et al*., 2011) and cucurbits in Taiwan (Knierim et al., 2010). New Poleroviruses have been reported as infecting chickpea and faba beans in Ethiopia (Abraham *et al*., 2006), peas and

Faba bean in China (Zhou *et al*., 2012), chickpeas in Sudan and Lentils in Ethiopia (Abraham *et al*., 2008).

More recently, a novel polerovirus, tentatively named Phasey bean mild yellows virus (PhBMYV) was identified as infecting peas (Wilson *et al*., 2012), *Trifolium subterraneum* (subterranean clover), *Cicer arietinum* (chickpea) and *Macroptilium lathyroides* (phasey bean) in Australia (Sharman *et al*., 2016). The virus is transmitted by the aphid vector, *A. craccivora* and induces stunting and mild yellowing symptoms in infected plants. Being a new virus, it is imperative that studies relating to its host range and full genome identification and the development of specific primers for its identification be carried out. This current research sought to identify these to enable informed decisions to be made regarding mitigation strategies for the management of the disease.

3 CHAPTER THREE: Impact of groundnut rosette disease on nutritive value and elemental composition of four varieties of peanut (*Arachis hypogaea*)

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Published in: Annals of Applied Biology 168: 400 – 408

3.1 Abstract

Proximate and elemental composition of four peanut genotypes infected with groundnut rosette disease (GRD) was examined. Moisture and ash content generally decreased while fat and energy content increased in seeds from diseased plants. Protein and carbohydrate varied between seeds of diseased and healthy plants of the different varieties with no consistent pattern. Instrumental neutron activation analysis of 10 elements within leaves, stems and seeds showed elevated levels of K, Al and Cl in leaves, stems and seeds in at least three of the four varieties infected with GRD while Na was decreased in stems but increased in seeds. While significant differences were found, Mg, Mn, Ca and Zn did not show any consistent change with respect to plant part or genotype, between diseased and healthy plants. V and Fe were found at low levels in leaves and stems and not detected in seeds. This represents the first report on the effect of GRD on the nutritive quality of peanuts.

3.2 Introduction

Peanut (*Arachis hypogaea* L.) also known as groundnut is a leguminous, nitrogen-fixing plant that is well suited to tropical, subtropical and warm temperate regions (Hammons, 1994). It is the world's 13th most important food crop, 6th most important source of edible oil and 3rd most important source of vegetable protein (Waliyar *et al.*, 2007). In Ghana, the crop is widely grown in the three northern regions for both home consumption and sale, with women actively involved in the harvest, processing and marketing (Masters *et al.*, 2013). Peanut seeds contain 44–56% oil, 22–30% protein (on a dry seed basis), 9.5–19.0% total carbohydrates (soluble and insoluble) and are a rich source of minerals (phosphorus, calcium, magnesium and potassium) and vitamins (E, K and B group). Relative to other staple crops, peanut is less expensive to grow and more nutritious making available nutrients that are insufficiently consumed by those most vulnerable to malnutrition (Savage and Keenan, 1994). It is also used as an ingredient in many traditional dishes and snacks as a major source of energy, protein, vitamins and minerals. Furthermore, the haulms (leaves and stock) are utilised as fodder and the cake (formed after the oil extraction) is a high-protein animal feed.

Despite the great potential of the crop to meet the nutritional requirements of the poor and vulnerable, its production has been constrained by several factors of which diseases are the most significant. Groundnut rosette disease (GRD), caused by synergistic interaction between *Groundnut rosette assistor virus* (GRAV): genus *Luteovirus*, *Groundnut rosette virus* (GRV): genus *Umbravirus* and its satellite RNA (SatRNA), is the most devastating disease of peanut in sub-Saharan Africa and is largely responsible for annual peanut yield loss worth over US$150 million (Waliyar *et al.*, 2007). Disease, besides reducing yield, may also affect the nutritive quality of the produce. Mosaic symptoms and/or necrotic lesions caused by systemic viral infection are indicative of structural changes in the chloroplasts, altered carbon metabolism and the accumulation of starch grains. In GRD, two predominant symptom types

are observed; ‘chlorotic’ and ‘green’ rosette. The expression of either form of the symptoms in young plants results in severe stunting due to shortened internodes and reduced leaf size (Naidu *et al.*, 1999a). In Ghana, both forms are present, but green rosette is more common. Changes in nutrient and mineral content of plant tissues following virus infection have been reported for a wide range of crops and pathogens. These are briefly reviewed in this article. Such changes may mean that a particular mineral could be absorbed in excess, leading to toxicity or may be absorbed in quantities lower than required as a result of viral infection. Although several studies have reported on GRD, most attention has been given to the quantitative effect regarding yield losses. The impact of the disease on the nutritive quality of peanuts has received little or no attention. However, it is important to understand the effect the disease has on quality of peanuts as this determines the value of the crop on both local and international markets and the capacity to meet nutritional needs. This study focuses on the effect of GRD ‘green rosette’ on the nutritive as well as the elemental composition of four varieties of peanut grown under greenhouse conditions.

3.3 Materials and methods

3.3.1 Plant material

Four peanut genotypes obtained from the Crop Research Institute of the Council for Scientific and Industrial Research, Kumasi, Ghana, were used in this study. Three genotypes were commercial varieties with both Nkosuor and Otuhia having putative resistance to GRD and Yenyawoso regarded as susceptible. The fourth genotype was Sinkapoporigo; an unofficial variety commonly grown by Ghanaian farmers. This study was conducted at the Biotechnology and Nuclear Agriculture Research Institute of the Ghana Atomic Energy Commission, Accra.

3.3.2 Graft inoculation

Twenty seedlings of each genotype were raised in 2 L polythene bags filled with a steam sterilised loam soil in an insect-proof screen house. They were side-grafted 3weeks after emergence with GRD-infected peanut scions collected from a single field-grown plant. GRAV infection of the plant from which the scions were taken, and the absence of infection with *Groundnut ringspot virus*, *Peanut mottle virus*, *Groundnut bud necrosis virus* (GBNV) and *Cowpea mild mottle virus* was confirmed by enzyme-linked immunosorbent assay (ELISA). Control plants were grafted with healthy scions obtained from plants raised in an insect-proof screen house. The grafted seedlings were covered with polythene bags for 1 week and then grown within an insect-proof screen house and observed for disease symptoms. Daily temperature and humidity were measured using a thermo-hygrometer.

Infection of the inoculated plants was confirmed by ELISA testing for the presence of GRAV at 30 days after inoculation when disease symptoms had fully developed. Fully expanded leaves at the same positions (topmost, second and third topmost) on the plants were sampled. The test format used was the triple antibody sandwich (TAS) ELISA using a polyclonal antibody to a *Beet western yellows virus* isolate obtained from the DSMZ Plant Virus Collection as a capture antibody and the monoclonal antibody, SCRI 6 obtained from the James Hutton Institute, Scotland as the detecting antibody. TAS-ELISA was carried out as previously described by Rajeshwari *et al.* (1987). Leaf samples (0.5 g) were ground in 5mL of extraction buffer containing 20 g of polyvinylpyrrolidone, 2 g of ovalbumin, 1.3 g of sodium sulphite (anhydrous), 0.5 mL of Tween-20, 20.8 g of sodium chloride, 0.2 g of potassium dihydrogen orthophosphate, 1.15 g of disodium hydrogen orthophosphate and 0.2 g of potassium chloride per litre. A known GRD sample and healthy peanut were used as positive and negative controls, respectively. Absorbance values were measured at 405nm

using a spectrophotometer (Multiskan Ascent VI.25-Version 1.3.1). Samples with absorbance values more than twice that of the healthy controls were considered positive.

3.3.3 Proximate analysis

Analyses were carried out on the seeds harvested from GRD plants of each genotype and their respective controls to determine the ash, moisture, fat, protein, carbohydrate and energy content. Ash content was determined by weighing the remainder of a 5 g sample heated in a furnace at 550◦C for 16 h (AOAC, 2000). Moisture content was determined by drying 3 g of the samples in a hot-air oven at 105◦C for 3 h until a constant weight was achieved (AOAC, 2000). The crude fat content was determined following a Soxhlet Extraction procedure (AOAC, 2000). Fats were extracted from approximately 3 g samples with diethyl ether in a Soxhlet Apparatus at 80∘C for 14 h. Total protein was determined using a Kjedahl method (AOAC, 2000), with a nitrogen conversion factor of 5.46 (protein-nitrogen conversion factor for peanut). The carbohydrate content of the seeds was determined by difference, from the following equation: carbohydrate (%) =solids – (protein+fat+ash), where solids=100 – moisture content. The energy content of the seeds was estimated using the Atwater specific factor system (Food and Agricultural Organization, 2002) from the following equation: energy (kcal)=8.37 (fat)+3.47(protein)+4.07 (carbohydrates).

3.3.4 Elemental composition analysis using instrumental neutron activation analysis

Infected plants of the four peanut genotypes and their healthy controls were harvested and separated into leaves, stems and pods. The pods were sun-dried for 24 h and then shelled. Samples were rinsed with sterilised distilled water, freeze-dried and pulverised and the powdered samples stored in plastic bags at 4∘C prior to analysis. Samples (200 mg) and

control (National Bureau of Standards, Standard Reference Material 1572 *Citrus* leaves) were wrapped in a transparent polythene film, placed in irradiation capsules and heat sealed for neutron activation. Each powdered sample was assayed three times in separate plastic vials.

The samples and controls were irradiated at the Ghana Research Reactor-1 (GHARR-1; a miniature neutron source reactor) at the Ghana Atomic Energy Commission. GHARR-1 is operated at 15KW which generates a thermal flux of 5 × 1011n-cm−2s−1. The irradiation scheme was based on elements of interest and the half-life of the radionuclide. Short-lived radionuclides (Al, Ca, Cl, Mg, Mn and V) were irradiated for 2 min and the activated samples delayed for 2–5min and then counted for 10min. Medium-lived radionuclides (K, Na) were irradiated for 1 h, delayed for 24 h and then counted for 10 min. Long-lived radionuclides (Fe, Zn) were irradiated for 6 h and the activated samples delayed for 2weeks and then counted for 10 h. Radioactivity measurements of induced radionuclides were performed by a PC-based γ-ray spectrometry set-up consisting of an N-type High purity Germanium detector Model GR 2518 (Canberra Industries Inc. Meriden, CT, USA) coupled to a computer based multi-channel analyser via electronic modules. The relative efficiency of the detector is 40% and its energy resolution is 1.8 keV at a -ray energy of 1332 keV of 60Co source. Data acquisition and identification of γ-rays of product radionuclides were identified by their γ-ray energies via ORTEC MAESTRO-32 (Maestro for Windows model A65-B32, Version 6.05, Advanced Measurement Technology, Inc., Oak Ridge, TN, USA). Gamma spectrum evaluation and quantitative analysis of each sample and its control were done with multipurpose γ-ray spectrum analysis software; WinSPAN-2010 version 2.10 using relative comparator methodology.

3.4 Results

All four peanut genotypes used in this study succumbed to GRD and expressed typical green rosette symptoms (Waliyar *et al.*, 2007) these being shortening of the internodes leading to severe stunting, reduced leaf size, leaf distortion and mosaic (Fig. 3.1). Symptoms appeared 14 days after grafting and persisted throughout growth of the plants. The seeds obtained from the infected plants at harvest were shrivelled and smaller than those from healthy plants. TAS-ELISA confirmed the presence of GRAV in all inoculated plants while the controls tested negative. Nkosuor had the highest GRAV titre (0.628 ELISA absorbance value) which was significantly greater than Otuhia (0.451) and Yenyawoso (0.442). The differences in the virus titre among the genotypes did not influence symptom severity with all showing severe stunting and reduced leaf size. The proximate analysis of seeds from diseased plants of the four peanut genotypes and their respective controls is shown in Table 1. Moisture content was significantly reduced (P <0.01) in seeds from diseased plants of Nkosuor, Sinkapoporigo and Yenyawoso with the highest reduction observed in Sinkapoporigo (37.8%). In Otuhia, however, moisture increased (P <0.01) in seeds from diseased plants. Similarly, decreased ash content was observed in seeds of Sinkapoporigo and Nkosuor while an increase was seen in Otuhia. Protein content significantly increased (P <0.01) in the seeds of GRD-infected plants of Nkosuor (25.55%) and Otuhia (26.6%) but decreased in Sinkapoporigo. No significant differences in protein content were found in the seeds of Yenyawoso. Fat content increased significantly (P >0.01) in seeds of infected plants of all the genotypes except Nkosuor. Carbohydrate content decreased significantly in seeds of virus-infected plants of Otuhia (25.72%) and Yenyawoso (38.03%) but increased (P <0.01) in that of Nkosuor (41.07%) and Sinkapoporigo (28.51%) when compared with healthy seeds. With the exception of Nkosuor, all the seeds from virus-infected plants of the four varieties had

significantly higher energy content (P <0.01) than their respective controls, with the highest energy content associated with Sinkapoporigo (483.46 kcal).

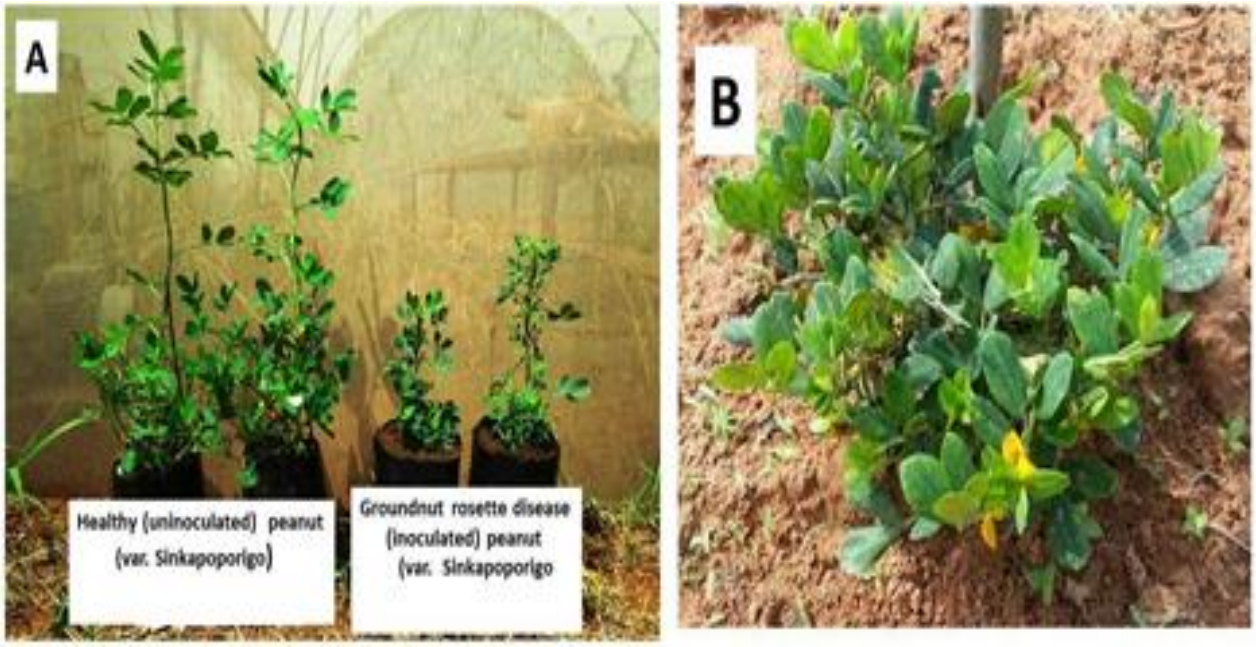

Figure 3.1 (A) Healthy and Groundnut rosette diseased peanut plants of the variety Sinkapoporigo. (B) Close-up of Groundnut rosette diseased peanut plant

Table 3.1 Proximate analysis of GRD-infected peanut seeds

Variety	Treatment	Mean ELISA absorbance values	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Carbohydrate (%)	Energy (kcal/100g)
Nkosuor	H	0.099 c	15.40 c	2.17 b	23.63 c	27.75 b	31.01 d	468.34 c
	D	0.628 a	12.20 e	1.95 d	25.55 b	19.23 d	41.07 b	439.60 e
Otuhia	H	0.135 c	20.39 b	1.78 e	21.70 d	18.00 e	38.13 c	401.29 g
	D	0.451 b	21.55 a	1.96 cd	26.60 ab	24.17 c	25.72 f	426.83 f
Sinkapoporigo	H	0.128 c	21.89 a	2.53 a	26.99 a	26.89 b	22.12 g	438.40 e
	D	0.493 ab	13.62 d	2.12 bc	25.73 b	29.61 a	28.51 e	483.46 b
Yenyawoso	H	0.115 c	9.67 f	2.53 a	23.64 c	20.23 d	43.92 a	452.37 d
	D	0.442 b	8.62 g	2.53 a	23.61 c	27.21 b	38.03 c	491.43 a
Pr value		*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.001*
LSD		0.152	0.921	0.161	1.066	1.226	1.921	7.399

D = diseased; H = healthy

Values in columns followed by the same letter are not significantly different (P >0.05) from each other according to the least significant difference test.

Pronounced changes in the mineral content of leaves, stems and seeds of virus-infected plants were observed (Tables 3.2, 3.3, and 3.4). For the macronutrients, potassium content increased significantly (P <0.01) in the infected tissues of all genotypes. In general, the concentration of K was lower in the seeds compared with the stems and leaves. The highest concentration of K was observed in the infected leaves of Sinkapoporigo (5640 mg/100 g). Calcium levels significantly increased in Nkosuor (leaves and seeds only), Yenyawoso (leaves and stems), Otuhia (seeds) and Sinkapoporigo (leaves and stems) and significantly decreased in Nkosuor (stems) and Otuhia (leaves and stems). Magnesium content increased significantly (P <0.01) in all infected tissues except for leaves of Otuhia and Yenyawoso and seeds of Otuhia which showed significant decreases, and seeds of Sinkapoporigo and Yenyawoso which showed no differences. The highest concentration of Mg was found in infected leaf samples of Sinkapoporigo (1090 mg/100 g).

With the micronutrients, chlorine content increased significantly (P <0.01) in the leaves, stems and seeds of virus-infected plants of the four varieties; however, a much lower Cl content was recorded in the seeds than in the leaves and stems. The highest concentration of Cl was found in infected stem of Otuhia (3450 mg/100 g). Aluminium content increased significantly (P <0.01) in the tissues of all varieties except within leaf and seed samples of Yenyawoso. The concentration of Al in the seeds was again, much lower than those found in the stems and leaves and ranged from 4.41 to 6.06 mg/100 g. Manganese content significantly increased (P <0.01) in the infected leaf samples of Sinkapoporigo and Nkosuor but decreased (P <0.01) in Otuhia and Yenyawoso. A significant decrease in Mn content was also found in infected stems of Nkosuor.

Table 3.2 Elemental compositions of leaves of four varieties of Groundnut rosette disease-infected and healthy peanuts

Variety/	Treatment[a]	Elemental composition (mg/100g)[b]									
		Mn	Al	Na	V	Cl	Fe	Zn	K	Ca	Mg
Sinkapoporigo	H	6.96 f	21.24 d	nd	0.046 cd	1482.7 f	nd	2.969 b	2258 e	2093 d	686.9 d
	D	14.76 a	34.54 a	25.52 b	0.090 a	2785.0 b	36.1 b	nd	5635 a	3560 a	108.7 a
Nkosuor	H	3.27 h	19.43 e	36.13 a	nd	1999.0 d	51.96 a	3.979 a	2489 d	1110 g	348.7 g
	D	8.20 d	26.13 b	14.63 c	0.049 c	2478.0 c	56.54 a	2.739 b	3115 b	2061 d	559.5 e
Otuhia	H	10.14 c	16.75 f	nd	nd	2811.0 b	nd	2.629 b	954 g	2362 c	885.4 b
	D	7.93 e	24.65 c	15.75 c	0.041 d	2904.0 a	nd	2.646 b	2746 c	1635 e	560.3 e
Yenyawoso	H	10.54 b	24.73 bc	nd	0.035 e	1860.0 e	nd	nd	1384 f	1336 f	720.8 c
	D	5.41 g	15.20 g	nd	0.063 b	999.2 g	24.47c	2.987 b	2135 e	3137 b	492.2 f
P value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD		0.139	1.429	2.605	0.0054	88.03	5.016	0.4473	162.78	143.61	32.82

[a] D = diseased, H = healthy

[b] nd = not detected.

Values in columns followed by the same letter are not significantly different ($p>0.05$) according to the least significance difference test.

Table 3.3 Elemental composition of stems of four varieties of Groundnut rosette disease-infected and healthy peanuts

Variety	Treatment[a]	Elemental composition (mg/100g)[b]									
		Mn	Al	Na	V	Cl	Fe	Zn	K	Ca	Mg
Sinkapoporigo	H	2.03 a	11.69 a	29.07 d	0.047 b	1676 b	nd	nd	2944 c	930 de	304.9 f
	D	2.55 ab	20.69 c	27.68 c	nd	1850 c	29.66 a	3.49 b	3947 a	1075 c	602.6 c
Nkosuor	H	8.66 d	21.00 c	54.10 e	0.046 b	1740 bc	39.94 b	2.64 a	1892 e	1933 a	599.4 c
	D	3.41 c	28.00 e	13.25 a	0.061 c	2891 d	53.04 c	4.16 c	3246 b	1454 b	737.8 b
Otuhia	H	2.98 bc	18.69 b	41.58 d	nd	3107 e	31.55 a	3.25 ab	1631 f	1428 b	466.8 d
	D	2.59 ab	24.35 d	29.36 c	0.043 b	3450 f	29.66 a	nd	3810 a	1101 c	1011.9 a
Yenyawoso	H	2.20 a	11.90 a	25.32 c	0.035 a	1275 a	nd	nd	2546 d	867 e	398.6 e
	D	2.55 ab	25.62 d	20.03 b	0.079 d	1678 b	52.69 c	2.72 a	3042 c	1018 cd	400.9 e
P value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD		0.366	0.822	1.388	0.0051	49.54	37.25	0.318	141.57	111.06	17.33

[a] D = diseased, H = healthy

[b] nd = not detected.

Values in columns followed by the same letter are not significantly different ($p>0.05$) according to the least significance difference test.

Table 3.4 Elemental composition of seeds of four varieties of Groundnut rosette disease-infected and healthy peanuts

Variety	Treatment[a]	Elemental composition (mg/100g)[b]									
		Mn	Al	Na	V	Cl	Fe	Zn	K	Ca	Mg
Sinkapoporigo	H	1.51 c	4.96 a	34.47 a	nd	23.93 a	nd	5.02 c	806.5 cd	300.5 a	206.6 cd
	D	2.01 e	5.85 b	42.08 a	nd	38.88 c	nd	4.36 ab	991.1 a	288.4 ab	215.4 bc
Nkosuor	H	1.43 bc	4.76 a	40.96 a	nd	26.27 a	nd	3.09 a	781.9 d	220.8 e	219.8 b
	D	1.33 ab	5.73 b	66.98 b	nd	53.25 d	nd	5.38 c	960.4 b	276.2 bc	248.5 a
Otuhia	H	1.36 abc	4.48 a	40.38 a	nd	36.40 bc	nd	4.61 c	693.2 f	181.4 f	201.2 d
	D	1.32 ab	4.87 a	69.42 b	nd	73.06 e	nd	4.41 ab	812.0 c	247.7 d	183.8 e
Yenyawoso	H	1.83 d	6.06 b	35.13 a	nd	25.65 a	nd	5.48 c	724.7 e	257.8 cd	243.2 a
	D	1.19 a	4.41 a	93.27 c	nd	31.87 b	nd	nd	812.2 c	238.3 de	241.1 a
P value		<0.001	<0.001	<0.001		<0.001		<0.001	<0.001	<0.001	<0.001
LSD		0.098	0.261	5.041		1.402		0.433	25.23	22.92	9.05

[a] D = diseased, H = healthy

[b] nd = not detected.

Values in columns followed by the same letter are not significantly different

In the seeds, Mn content significantly (P <0.01) increased in virus-infected samples of Sinkapoporigo but decreased in Yenyawoso. The highest content of Mn (14.76 mg/100 g) was found in the infected leaf sample of Sinkapoporigo. Sodium was not detected in the healthy leaf samples of Sinkapoporigo and Otuhia or in healthy and infected leaf samples of Yenyawoso. In Nkosuor, Na content was significantly reduced (P <0.01) in the infected sample (14.63 mg/100 g) compared with the healthy sample (36.13 mg/100 g). Sodium content significantly (P <0.01) decreased in stems of infected plants but increased (P <0.01) in the seeds of infected plants of all the genotypes, with the highest content (93.27 mg/100 g) associated with the seeds of Yenyawoso. Detection of Zn in this study was inconsistent with failure to detect this element in some leaves, stems and seed samples from both infected and healthy plants. The concentration of Zn decreased (P <0.01) in the infected leaf sample of Nkosuor (2.73 mg/100 g) but increased significantly (P <0.05) in the stems (4.16mg/100 g) compared with the healthy samples (2.64mg/100 g). The concentration of Zn was significantly increased (P <0.01) in infected seeds of Nkosuor (5.38 mg/100 g) but decreased in that of Sinkapoporigo (4.36 mg/100 g) and Otuhia (4.41 mg/100 g) while it was undetected in infected seeds of Yenyawoso. The concentration of vanadium significantly increased (P <0.01) in leaves of infected plants of Sinkapoporigo (0.090 mg/100 g) and Yenyawoso (0.063 mg/100 g), and stems of Nkosuor (0.061 mg/100 g) and Yenyawoso (0.079 mg/100 g). It was however, neither detected in some leaf and stem samples nor in any seed samples of the four varieties. Similarly, Fe was not detected in any of the seed samples or in some leaf and stem samples of both infected and healthy plants. Notwithstanding, an increased (P <0.01) Fe content was found in infected stem samples of Nkosuor (53.04 mg/100 g). Other samples showed no significant differences in Fe content.

3.5 Discussion

All four genotypes showed typical GRD symptoms with no marked differences in the symptoms expressed. Variability between varieties in GRD symptom expression has been previously reported (Naidu *et al.,* 1998). While ELISA testing only confirmed infection with GRAV, the presence of GRV and Sat RNA within symptomatic plants could be inferred as GRV and SatRNA are required for GRD symptoms (Murant *et al.*, 1988) and satellite-free isolates of GRV have not been found in nature (Murant, 1990). GRAV or GRV alone results in symptomless infection in peanuts. Proximate analysis of peanut seeds showed significant changes in the measured factors between infected and healthy plants, which often varied with genotype. The reduction in moisture content seen for three of the four genotypes may be due to premature loss of water from the hulls as a result of the disease, culminating in accelerated seed desiccation and shrivelling. A reduction in relative water content of pepper infected with *Tobacco mosaic virus* has also been reported (Pazarlar *et al.*, 2013). In contrast an increased moisture content of banana leaves associated with *Banana bunchy top virus* (BBTV) infection was shown (Hooks *et al.,* 2009) as was found for the infected Otuhia seeds. Diminished ash content as seen in two peanut genotypes has also been reported in virus-infected cowpea (Taiwo and Akinjogunla, 2006). The elevation of protein content of the seeds of virus-infected plants of Nkosuor and Otuhia may be attributed to the synthesis of virus coat protein and other virus-associated non-structural proteins. A marked increase in amino acid concentration has been reported previously in GRD-infected peanut (Singh and Srivastava, 1974). Several other reports note increased protein content as a result of virus infection, including within leaves of *Cucurbita pepo* infected with *Zucchini yellow mosaic virus* (Radwan *et al.,* 2007), soybean infected with *Soybean mosaic virus* (Cheema *et al.,* 2003) and tomato with a tospovirus infection (Sutha *et al.,* 1998). In contrast, similar reductions of protein content as seen in seeds from diseased plants of Sinkapoporigo have

been found in seeds from virus-infected cowpea plants (Taiwo and Akinjogunla, 2006), and in seed from peanut plants infected with *Peanut stripe virus* (PStV; Ross *et al.*, 1989).

Fat content increased in three of the four genotypes which contrasts with reports of reduced oil in peanut seeds following GBNV infection (Sunkad and Naik, 2013) and in oil content of soybean infected either singly or in double combinations with PMoV, *Cowpea chlorotic mottle virus*, *Soybean mosaic virus* and *Tobacco ringspot virus* (Demski and Jellum, 1975). The altered carbohydrate content observed in the seeds of diseased plants in this study could be due to alterations in photosynthetic metabolism and disorders in plant carbohydrate metabolism as a result of the infection (Gonçalves *et al.*, 2005). The disparities in carbohydrate content observed among the genotypes could be due to differences in their response to the disease. The reduction in carbohydrate content of seeds of virus-infected plants could be considered a secondary effect of the viral infection. Infection may have inhibited phloem transport, culminating in the accumulation of photosynthetic products in chloroplasts and a subsequent reduction of their supply from source leaves to seeds. The decrease in carbohydrate content could also be due to increased respiration that characterises virus-infected plants and the possible conversion of carbohydrates into amino acids, which are used for protein synthesis (Gupta *et al.*, 2010). Reduction in carbohydrate content in *Bean yellow mosaic virus*-infected bean has also been shown (Mali *et al.*, 2000). In contrast, increased carbohydrate content of peanut seeds following PStV infection (Ross *et al.*, 1989), cowpea seeds following virus infection (Taiwo and Akinjogunla, 2006) and sugarcane leaves as a consequence of *Sugarcane yellow leaf virus* infection (Gonçalves *et al.*, 2005) have been reported. The accumulation of carbohydrates in the virus-infected seeds observed in this study could also be due to more efficient translocation of assimilate such as was observed with *Cocoa swollen shoot virus*-infected cocoa (Adomako and Hutcheon, 1974) or the inactivation of carbohydrate-utilising enzymes due to the infection, resulting in reduced

breakdown of carbohydrates. The higher energy content associated with the seeds from the virus-infected plants in this study could be due to the increased fat content.

The mineral content of stems and seeds of the tested peanut genotypes also varied with GRD infection. Plant disease impairs physiology, including nutrient uptake, assimilation, translocation from the root to the shoot and utilisation. GRD affected plants may have altered membrane permeability (Huber and Graham, 1999), resulting in increased or decreased uptake of particular elements. Furthermore, the impaired uptake of elements by plant tissues may result in nutrient interactions leading to changes in their concentrations (Fageria, 1983). Within the measured macronutrients, increased potassium content observed in all genotypes is consistent with a prior study comparing sugar beet infected with *Beet yellows virus* (Clover *et al.*, 1999) but contrasts with reports of decreased K in peanut seed infected with PStV (Ross *et al.*, 1989) and virus-infected millet (Kandhasamy *et al.*, 2010). Calcium and magnesium content both increased and decreased in the peanut genotypes tested. There are similar contrasting prior reports of changes in Ca content in virus-infected plants. Decreased Ca has been found in tiger millet infected with Ragi mottle streak virus (Kandhasamy *et al.*, 2010), while peanut seed showed no change in Ca following PStV infection (Ross *et al.*, 1989). Reported changes in Mg concentration of virus-infected plants are also inconsistent with increased Mg in virus-infected peanut (Ross *et al.*, 1989) and tropical soda apple (Overholt *et al.*, 2009) while finger millet (Kandhasamy *et al.*, 2010), and *Solanum nigrum* (Takács *et al.*, 2001) showed decreased Mg content. Potassium has an antagonistic effect on the absorption of Ca2+ and Mg2+ at higher concentration and this depends on plant species and environmental conditions. It is thus possible that the higher concentration of K observed in this studymay have led to the relatively lower concentrations of Ca and Mg in the plant tissues (Fageria, 1983). The increase in chlorine and aluminium content following GRD

infection agrees with Ikram-ul-Haq *et al.* (2014) whom observed a trend for greater Cl content in leaves of banana infected with BBTV. Variability in the response of virus infection on micronutrient content of plant tissues has been previously reported. Virus infection resulted in increased (Overholt *et al.*, 2009) or decreased (Takács *et al.*, 2001) Mn content in *Solanum* spp. or no effect in grapevine (Moutinho-Pereira *et al.*, 2012). Similarly, both reduction (Takács *et al.*, 2001; Ashfaq *et al.*, 2014) and increase in Na (Clover *et al.*, 1999; Ikram-ul-Haq *et al.* (2014)) and reduction (Takács *et al.*, 2001), increase (Overholt *et al.*, 2009; Ashfaq *et al.*, 2014) or no effect in Zn content (Moutinho-Pereira *et al.*, 2012) of virus-infected plants have been reported. Similarly, reduced (Kandhasamy *et al.*, 2010) or increased (Ashfaq *et al.*, 2014) Fe content of virus-infected plants has been reported. Vanadium increased in foliar parts of some of the varieties. Acute vanadium poisoning was found in sheep following experimental daily feeding of diets containing 400–800mgkg−1 (40–80mg 100 g−1) ammonium metavanadate (Hansard *et al.*, 1978). Furthermore, higher accumulation of V in soils, water and vegetables poses potential effects to human and animal health (Vachirapatama *et al.*, 2011). However, V was not detected in the nuts which are the parts consumed by humans and the concentration of V observed in the stems and leaves which are used as fodder for livestock, are too low (0.035–0.090 mg/100 g) to have any deleterious effects when fed to animals. Despite the importance of peanut as a source of energy and nutrients for people and livestock of sub-Saharan Africa, the impact of disease on the nutritive quality of peanuts has received little or no attention. This study has detailed changes in nutritive qualities of peanut leaf, stem and seed tissues when plants succumb to GRD. Significant changes as a result of infection were found which varied with variety. In the majority of varieties, energy content increased with virus infection. While disease results in profound yield loss, such changes in quality can affect the nutritive benefits of the produce.

Increased energy content may compensate to some extent for reduced yield by providing nutritional boost. Concerns over changes in elemental composition that may render stock or human feed toxic following virus infection are allayed.

3.6 Acknowledgements

The authors wish to thank Australian Agency for International Development (AusAID) for the funding. We are also grateful to the Tasmanian Institute of Agriculture and the Biotechnology and Nuclear Agriculture Research Institute of the Ghana Atomic Energy Commission (GAEC) for their support in terms of facilities.

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Appiah, A. S., Offei, S. K., Tegg, R. S., Wilson, C. R., 2016. Varietal response to groundnut rosette disease and the first report of Groundnut ringspot virus in Ghana, Plant disease, 100(5),946-952 https://doi.org/10.1094/PDIS-07-15-0838-RE

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Appiah, A. S., Sossah, F. L., Tegg, R. S., Offei, S. K., Wilson, C. R., 2017. Assessing sequence diversity of Groundnut rosette disease agents and the distribution of Groundnut rosette assistor virus in major groundnut-producing regions of Ghana, Tropical plant pathology, 42(2), 109–120
http://dx.doi.org/10.1007/s40858-017-0140-x

6 CHAPTER SIX: Genomic analysis and transmission of recombinant Phasey bean mild yellows virus isolates from Queensland and Tasmania

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6.1 Abstract

The Poleroviruses include many species of economic importance, affecting many plant species and causing significant losses. Furthermore, the relatively frequent discovery of new Poleroviruses and a propensity for genomic recombination has identified this genus as one providing numerous emerging viruses of importance. In this study, the full genome a Queensland isolate of the novel Polerovirus, tentatively named Phasey bean mild yellows virus (PhBMYV) was determined. The full genome consisted of six open reading frames (ORFs), with their respective nucleotide and putative protein sequences sharing close identity to two previously reported isolates from New South Wales (NSW) and Western Australia (WA), except within the RNA-dependent-RNA-polymerase (RdRp) and Coat protein-Read through (CP-RT) genomic regions. The RdRp shared ~63% amino acid identity with the NSW and WA isolates while the CP-RT was highly divergent sharing only 33-34%, being more closely related to that of *Chickpea chlorotic stunt virus* (53%). Recombination analysis suggested this novel virus isolate is likely to have been derived from a recombination event with an exchange point identified at the boundary of the CP and CP-RT genomic regions. In an experimental host range study, the virus was successfully transmitted to Phasey bean, pea and chickpea plants using both aphid vector and graft transmission methods. Symptoms observed were severe and included yellowing, stunting and reduced leaf size. Based on the results of this study, PhBMYV QLDCL 16 is suggested as a genetic variant of PhBMYV.

6.2 Introduction

Legume production is an important venture in Australia with the industry currently contributing about AUD$1.6 billion per year to the Australian economy (Australian Food News, 2016). These include lentils, chickpeas, faba beans, field peas, lupins and mungbeans (Pulse Australia, 2015). Virus diseases pose a serious risk to legume production. Several virus species have been identified from legume crops in Australia (Latham and Jones, 2001a; Schwinghamer and Schilg, 2003; Thomas *et al*., 2004). Of these, *Alfalfa mosaic virus* (Bromoviridae, genus Alfamovirus) (AMV), *Cucumber mosaic virus*, (Bromoviridae, genus Cucumovirus) (CMV), Bean yellow mosaic virus and *Pea seedborne mosaic virus* (Potyviridae, genus Potyvirus) (BYMV and PSbMV), (Jones and Coutts, 1996) and *Subterranean clover stunt virus* (SCSV) (Nanovirus) (Wilson *et al*., 2012) are the most commonly found. However, many crop surveys across Australia have confirmed the presence of viruses belonging to the the family Luteoviridae in pulse crops, whilst sometimes less abundant, often resulting in severe disease. These include *Turnip yellows virus* (TYV) (genus Polerovirus) (Wilson *et al*., 2012), *Bean leaf roll virus* (genus Luteovirus) (BLRV) and *Beet western yellows virus* (genus Polerovirus) (BWYV) (Schwinghamer *et al*., 1999; Latham and Jones, 2001a; Freeman and Aftab, 2001; Van Leur *et al*., 2003; Aftab *et al*., 2005). The Luteoviridae is made up of three genera; Luteoviruses, Enamoviruses and Poleroviruses. They are all transmitted in persistent (circulative, non-propagative) manner by aphid vectors (Duffus, 1977). Their genomes are positive-sense ssRNA of about 1.8-2 x 106 Mr (5.5-6.0 kb) with a genome-linked protein (VPg) covalently attached at the 5' end of the RNA molecule which is not polyadenylated at the 3'-end (Mayo *et al*., 1982; Murphy *et al*., 1987). The 3' ends do not appear to possess tRNA-like structures such as those associated with other plant viral groups (Miller *et al*., 1988). The genomes of the Luteoviridae contain five to six open reading frames (ORFs) (Pfeffer *et al*., 2002).

The Polerovirus genus is particularly associated with the emergence of new viral diseases worldwide due to the existence of high genetic diversity and putative propensity for genetic recombinations (Moonan *et al*., 2000; Stevens *et a*l., 2005; Lotos *et al*., 2016), and are known to cause major yield losses in vegetable and arable crops. Recently, novel Poleroviruses have been reported in chickpea and faba bean in Ethiopia (Abraham *et al*., 2006), cotton in Australia and Argentina (Ellis *et al*., 2013; Distefano *et al*., 2010), strawberry in Canada (Xiang *et al*., 2015), cabbage, maize and brassicas in China (Zhang *et al*., 2014; Wang *et al*., 2016; Xiang *et al*., 2011) and cucurbits in Taiwan (Knierim *et al*., 2010). In a recent survey of vegetable crops in Tasmania, a novel polerovirus was identified as infecting peas (Wilson *et al*., 2012). The virus has subsequently been found in *Trifolium subterraneum* (subterranean clover) in Western Australia (WA), *Cicer arietinum* (chickpea) in New South Wales (NSW) and *Macroptilium lathyroides* (phasey bean) in Queensland (QLD) and has been tentatively been named Phasey bean mild yellows virus, PhBMYV (Sharman *et al*., 2016). The emergence of novel viruses and the diseases they cause; many of which are yet to be identified, present a major constraint to the production of food crops worldwide. The identification of a novel Polerovirus in Australian legumes thus necessitates studies on its epidemiology and genome characteristics. Here we describe virus hosts, confirm aphid transmission, obtain full and partial genomic sequences of new isolates, and compare genome sequences for evolutional recombination and to develop virus- specific primers for detection and diagnosis of PhBMYV; a pre-requisite for making informed decisions regarding mitigation strategies for the management of the disease.

6.3 Materials and Methods

6.3.1 Transmission studies using Queensland isolate of PhMYV

An isolate of PhBMYV from a naturally infected Phasey bean plant and a colony of cowpea aphids (*Aphid craccivora*), its putative vector were obtained from the Department of Agriculture and Fisheries, Brisbane, Queensland, Australia. The virus-infected Phasey bean plant was maintained under glasshouse conditions (20±2^{o}C) while the aphids were maintained on cowpea plants in a controlled environment cabinet at 28^{o}C with a 16 h photoperiod.

All transmission tests were carried out in an insect-proofed glasshouse. Host plants tested belonged to the Leguminaceae (pea; cultivars Greenfeast, Snow pea, Sugar snap, Dwarf pea and TIA – a locally sourced cultivar), chickpea, cowpea, peanut, Faba bean and bean), Solanaceae (tomato, eggplant and capsicum), Asteraceae (lettuce) and Cucurbitaceae (zucchini and cucumber). Adult aphids were given a 24 hr acquisition access period (AAP) on leaves from a PhBMYV-infected plant and then individually transferred to two-week old receipient plants. About 10 – 15 adult aphids were used per plant. Eight to 15 plants of each test plant were aphid inoculated. A further ten plants were graft-inoculated with a scion from infected Phasey bean plant. Aphids fed on healthy Phasey bean plants were also transferred to ten test plants and healthy scions were grafted onto five healthy plants to serve as controls. The aphids were allowed 48 hr inoculation access period (IAP) and then killed with a pyrethrum-based insecticide. Inoculated plants were grown in an aphid-proof glasshouse for 4 – 8 weeks and observed for symptoms. Virus infection was tested 2-4 weeks post-inoculation by reverse-transcription polymerase chain reaction (RT-PCR).

6.3.2 RNA isolation and Reverse-transcription polymerase chain reaction (RT-PCR).

Total RNA was extracted from leaf tissues of both inoculated and non-inoculated plants using a PowerPlant RNA Isolation Kit (MO BIO Laboratories Inc. California, USA) following the manufacturer's instructions, 50mg of leave samples initially homogeniseed at 6.5 rpm for 45 sec in a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Model 6004-500, USA). The isolated RNA was kept at -80^{o}C used.

Complementary DNA (cDNA) was synthesized in a 25 µl reaction mix using the iScript Reverse Transcription Supermix (BIO RAD, California, USA). PCR was carried out in a 20 µl reaction volume containing 10 µl of HotStarTaq Plus Master mix comprising 1 unit HotStartTaq Plus DNA polymerase, 1x PCR buffer and 200µM of each dNTP), 0.75µl each of forward and reverse primers (10 µM), and 1 µl of template cDNA. PCR was performed in Eppendorf Mastercycler, Hamburg, Germany, using a thermocycle of 30 cycles of 30 s at 94°C, 60 s at 58°C, and 3 min at 72°C, preceded by an initial denaturation for 1 min at 94°C and a final extension at 72°C for 10 min. Amplified products were electrophoresed on 1% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, CA, USA), and viewed with the Safe Imager Transluminator (Invitrogen, CA, USA).

6.3.3 Next-generation sequencing (NGS) of QLD isolate of PhBMYV

RNA was isolated from 6g of infected leaves and precipitated by addition of 0.1 volume 3M sodium acetate and 2 volumes 100% ethanol followed by centrifugation at 13,000 rpm for 1 minute. Pelleted RNA was sent to Macrogen (Seoul, South Korea) for library preparation and 100-bp paired-end sequencing on an Illumina HiSeq2000.

6.3.3.1 Analysis of NGS data

The reads were first trimmed and then assembled into contigs using the de novo assembly function in Geneious 8.1.8 (Biomatters). Contigs were sorted by length (minimum length 500) and the longest subjected to BLAST searches (Altschul *et al*., 1990). In addition, the reads were also mapped to reference genomes obtained from GenBank (KT962999 for NSWCP15 and KT963000 for ESPCL15); as variants of the same virus. Mapping was performed with minimum overlap of 10%, minimum overlap identity of 80%, allow gaps of 10% and fine tuning set to iterate up to 10 times (Kehoe *et al*., 2014). A consensus between the contigs of interest and mapping was created in Geneious by alignment with Clustal W. The resulting sequences were compared with the reference sequences used in the mapping process.

The complete sequence obtained was aligned with the two reference genomes using ClustalW (Thompson *et al.*, 1994). Construction and confirmation of the genome sequence determined by NGS was done by PCR amplification of overlapping genome sequences and Sanger sequencing. PhBMYV primers were designed on the consensus sequence using the Primer3 function implemented in Geneious 8.1.8 (Biomatters) (Table 6.1). Complementary DNA (cDNA) was synthesized from a total RNA extract in a 25 µl reaction mix using the iScript Reverse Transcription Supermix (BIO RAD, California, USA) following the manufacturer's protocol. The cDNA was then used for PCR using reaction mixes and thermocycle profile as previously described, and amplicons viewed as before. DNA bands of interest were excised from the gel and purified using QIAGEN gel extraction kit (QIAGEN GmbH, Hilden, Germany). The purified products were then sequenced in both directions at the Australian Genome Research Facility, Melbourne, Australia. The resulting sequences were edited in Geneious 8.1.8 (Biomatters) and then mapped to the sequence obtained by the NGS. The sequences were then edited to ensure complete homology and the consensus sequence extracted.

Table 6.1 Oligonucleotide primers used for detection and sequencing of the virus genome

Primer	Sequence	Amplicon product size
PhBV1F	5'-CAACTACCGCCGCTCCCAGT- 3'	
PhBV842R	5'-GTTACCCCTACCTTGTGGGC-3'	842bp
PhBV197F	5'-GGCGCAAAAGCTGAACTGAA-3'	
PhBV1679R	5'-ATTGCTCTCACGTGGCTTGA-3'	1472bp
PhBV739F	5'-ATACAGCTCTGGAGGGTCGT-3'	
PhBV2032R	5'-AGAGGCCTCGGAGATGAACT-3'	1293bp
PhBV1660F	5'-TCAAGCCACGTGAGAGCAAT-3'	
PhBV2760R	5'-TGACCCAGCACAAAGTTGGT-3'	1100bp
PhBV2741F	5'-ACCAACTTTGTGCTGGGTCA-3'	
PhBV3919R	5'-GCCCGGTTGTCAACCCGGAT-3'	1178bp
PhB7F	5'-GATCCTTGTGCAAGTTTGTT-3'	
PhB455R	5'-GAATGAGACCTTTGTAAGTA-3'	467bp
PhB477F	5'-GATCTGTGGGGGCTCGCAT- 3'	
PhB830R	5'-TTCCTGGAAAATTTTCCCCAG -3'	373bp
PhB972F	5'-GTCCGCCTGTTTAATGGAAC-3'	
PhB1994R	5'-TTGGCAATCCATTGTTGCCGC-3'	1042bp
PhB4354F	5'-TTCGTTCACCTCTTTGACAAAG-3'	
PhB5789R	5'-ACTTGCCCATCAGTAGACAGG-3'	1455bp

6.4 Genome sequencing of Tasmanian isolate of Phasey bean mild yellows virus

The primer pairs (Table 6.1) were then used to amplify genomic sequences from a Tasmanian isolate of PhBMYV collected from an infected field pea sample in 2012 (Wilson *et al*. 2012). Total RNA extraction, cDNA synthesis, PCR and Sanger sequencing were done as previously described.

6.5 Genome analysis

Prediction of putative open reading frames (ORFs) using ORF finder and genome annotation were done in Geneious 8.1.8 (Biomatters, Auckland, NZ). To investigate potential

recombination events that might have occurred within the genome, the aligned sequences were subjected to recombination detection analysis using the Recombination Detection Program v.4.16 (RDP4) (Martin *et al*., 2015) with default settings. Potential recombination events (PREs) detected by the RDP were further checked with other recombination detection methods such as GENECONV (Padidam *et al*., 1999), BOOTSCAN (Martin *et al*., 2005), MaxChi (Maynard Smith, 1992) and CHIMAERA (Martin *et al*., 2005); implemented in RDP4. A breakpoint map showing the positions of all clearly identified breakpoints for a set of unique PREs was constructed.

6.5.1 Phylogenetic analysis

The complete genomic sequence of the QLD isolate of PhBMYV was aligned with other Luteovirus genomes retrieved from the Genbank (Table 6.2) including the PhBMYV isolates from NSW, NSWCP15 (KT962999) and WA, ESPCL15 (KT963000) using ClustalW (Thompson *et al*., 1994). The complete genome, and derived amino acid sequence data for individual open reading frames were subjected to phylogenetic analysis using the Neighbor-joining method using the Tamura-Nei model with a bootstrap value of 1000. Phylograms were visualised using TreeView (Page, 1996). Additionally, the PhBMYV isolates from QLD, NSW, WA and TAS were aligned, trimmed and used in phylogenetic analysis as described.

Table 6.2 List of Luteovirus sequences retrieved from the GenBank for this study

Virus	Acronym	Accession Number	Reference
Polerovirus			
Beet chlorosis virus	BChV	NC_002766.1	Hauser *et al*., 2002
Beet mild yellowing virus	BMYV	NC_003491.1	Guilley *et al*., 1995
Beet western yellows virus isolate Rouen 1	BWYV	KU521325.1	Hehn *et al*., unpublished
Carrot red leaf virus	CtRLV	NC_006265.1	Huang *et al*., 2005
Cereal yellow dwarf virus-RPS RPV Mex-1	CYDV-	AF235168.2|	Beckett & Miller

	RPS		unpublished
Cereal yellow dwarf virus - RPV	CYDV-RPV	L25299.1	Vincent *et al*., 1991
Chickpea chlorotic stunt virus	CpCSV	NC_008249.1	Abraham et al., 2006
Cucurbit aphid-borne yellows virus	CABYV	LC082306.1	Yoon et al., unpublished
Melon aphid-borne yellows virus	MABYV	NC_010809.1	Xiang *et al*., 2008
Potato leafroll virus isolate PLRV-IM	PLRV	KC456052.1	Li et al., unpublished
Sugarcane yellow leaf virus	ScYLV	NC_000874.1	Moonan *et al*., 2000
Tobacco vein distorting virus	TVDV	NC_010732.1	Mo *et al*., 2010
Turnip yellows virus	TuYV	NC_003743.1	Garcia *et al*., 1993
Phasey bean mild yellows virus isolate ESPCL15	PhBMYV	KT963000.1	Sharman *et al*. 2016
Phasey bean mild yellows virus isolate NSWCP15	PhBMYV	KT962999.1	Sharman *et al*. 2016
Luteovirus			
Barley yellow dwarf virus - PAV	BYDV-PAV	NC_004750.1	Miller *et al*., 1988
Bean leafroll virus	BLRV	NC_003369.1	Domier *et al*., 2002
Soybean dwarf virus	SbDV	NC_003056.1	Terauchi *et a*l., 2001
Enamovirus			
Pea enation mosaic virus-1	PEMV-1	NC_003629.1	Demler *et al*., 1991

6.6 Results

6.6.1 Virus transmission studies

Successful transmissions of PhBMYV from infected Phasey plant using with both *Aphis craccivora* as a vector or by stem grafting were achieved into healthy Phasey bean, pea and chickpea receipient plants. Phasey bean (Fig. 6.1), pea and chickpea (Fig. 6.2) plants showed stunting with severe yellowing and reduced leaf size. Tomato, eggplant, capsicum, lettuce, bean, cowpea, cucumber, zucchini, peanut and faba bean plants inoculated with the virus by either aphid vector or grafting remained symptomless and the virus was was not detected in the receipient plants. Infections of the inoculated Phasey bean, pea and chickpea plants were confirmed by RT-PCR and sequencing of the resultant amplicons.

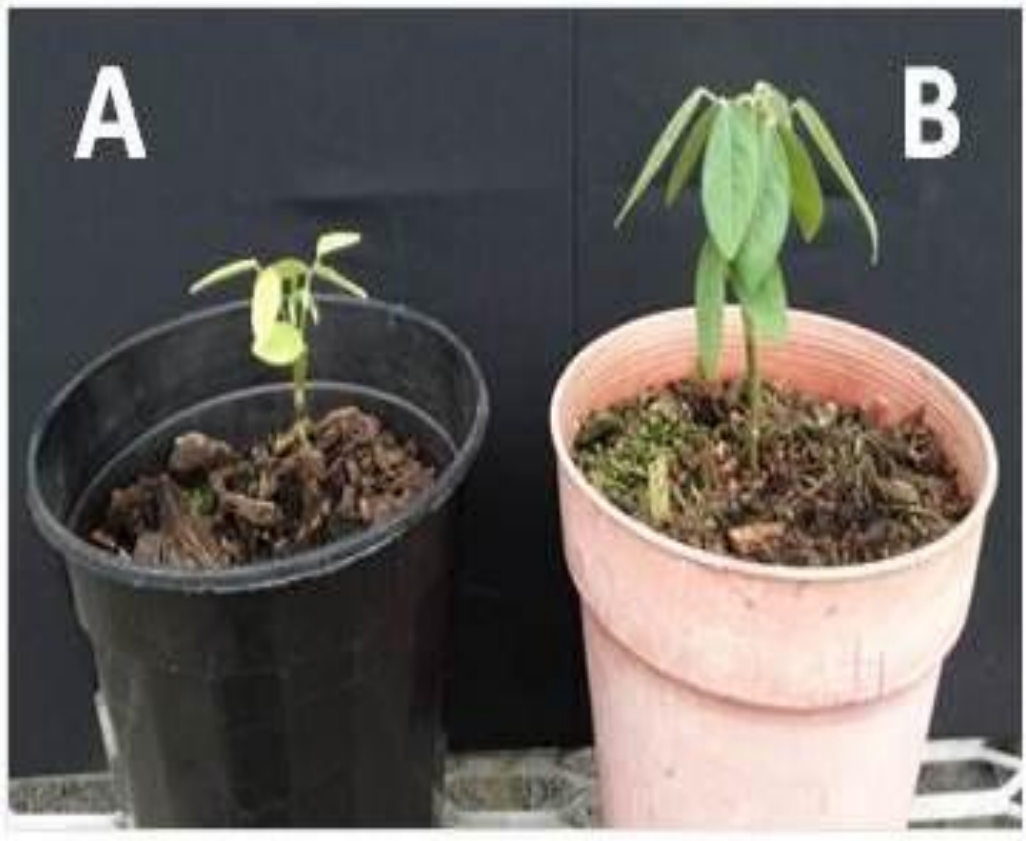


Figure 6.1 Phasey bean plants; **A**: PhBMYV-inoculated plant showing stunting, yellowing and reduced leaf size and **B**: Uninoculated Phasey bean plant.

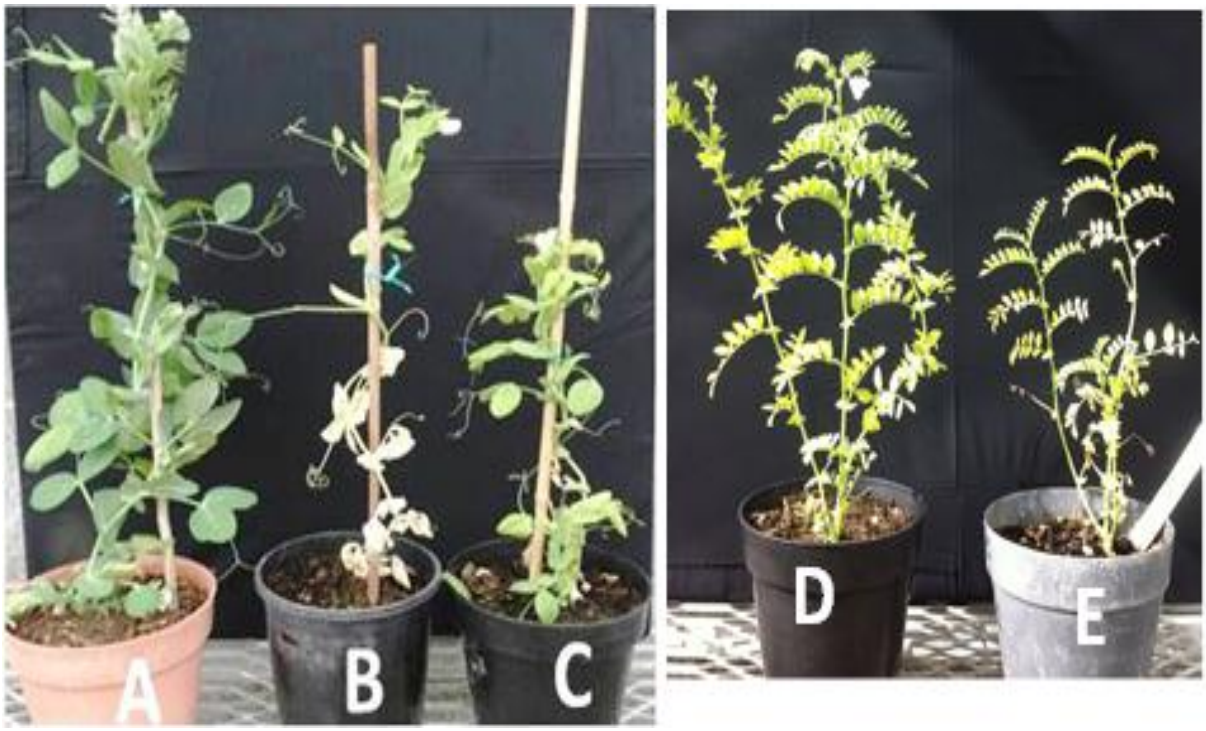


Figure 6.2 Phasey bean mild yellows virus-inoculated plants: **A.** Healthy pea **B.** Aphid-inoculated pea **C.** Graft-inoculated pea **D.** Healthy chickpea **E.** Aphid-inoculated chickpea.

6.6.2 Full length genome analyses of PhBMYV isolate QLD CL 16

From the single PhBMYV sample, 9,871,832 raw reads were obtained following NGS after trimming. Following de novo assembly in Geneious, 20,000 contigs ranging from 150 to 3, 000 nt were obtained. Contigs of interest were those between 500 and 3000 nt. After mapping to reference genomes in Geneious, the lengths of the consensus sequences were 5,867 nt for NSWCP15 (49,335 reads assembled) and 5,909 nt for ESPCL15 48,283 reads assembled). A full genome sequence of QLD isolate was derived form the consensus mapped to the ESPCL15 sequence. Oligonucleotides primers designed on the QLD PhBMYV consensus sequence were used in RT-PCR and are given in Table 6.1. Sanger sequencing of PCR amplicons was used to confirm the genomic sequence derived from NGS. The final sequence was the consensus of the full sequence from the NGS and the Sanger sequencing. The 5,868

nt complete genomic RNA of the QLDCL16 is shown in Fig. 6.3. Thirty (30) ORFs were predicted from the genome of which six (ORF0, ORF1, ORF2, ORF3, ORF4 and ORF5 were identified as typical of Poleroviruses. The overlapping region of ORF1 and ORF2 is ~578 nt long, while the intergenic region between ORF2 and ORF3 is ~195 nt.

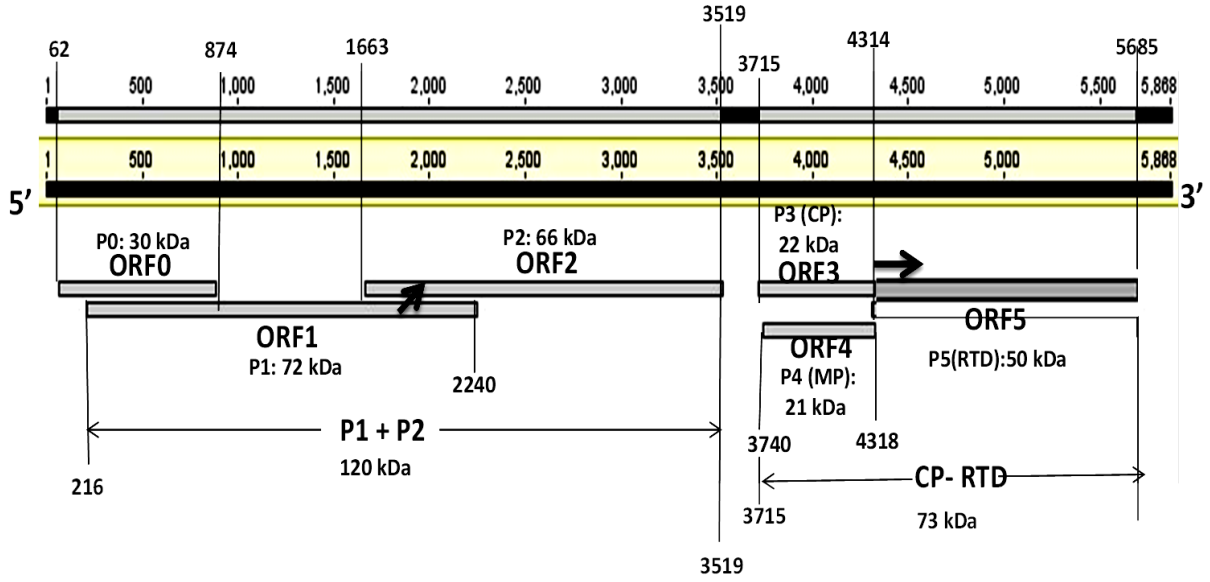


Figure 6.3 Schematic representation of the genome organisation of PhBMYV QLD isolate. The sizes of the deduced gene products are written either above or below the name of the ORF.

The predicted amino acid (aa) sequences of the six ORFs showed significant variation between the PhBMYV QLDCL 16 and the other members of the family Luteoviridae (Table 6.3). Sequence identities for the various proteins are as follows; P0 (09 – 97%), P1 (07 – 90%), P1 + P2 (06 – 61%), P3 (29 – 97%), P4 (26 – 93%), and P3+P5 (29 – 53%). The QLD isolate shared close aa identity to the NSW, WA and TAS isolates for all the proteins except P1 + P2 (52 - 61%) and P3+P5 (33-34%). The closest to the P3 + P5 protein of the PhBMYV QLDCL 16 was that of CpCSV sharing identity of 53%. The P4 protein shared aa sequence

identity of 67%, 68% and 69% with CABYV, PhBMYV TAS 12 and MABYV respectively. With the exception of PEMV-1, the P3 proteins appear less variable.

6.6.3 Analysis of partial sequence of Tasmanian isolate of PhBMYV

A 4300 nt partial sequence spanning the entire ORF0, ORF1, ORF2 and segments of ORF3 and ORF4 were obtained by using RT-PCR and Sanger sequencing. Nucleotide alignment of this segment with other PhBMYV isolates showed nucleotide identity of 96%, 94% and 90% with NSWCP 15, QLDCL 16 and ESPCL 16 respectively. The predicted amino acids sequences for ORF0, ORF1, ORF2, ORF3 and ORF4 of the Tasmanian isolate showed significant variation compared to the NSW, WA and the QLD isolates. For the P0 aa sequence, the TAS isolate shared 84% (WA), 74% (NSW), and 97% (QLD). The P1 aa sequences were less variable with the TAS isolate sharing 95%, 85% and 89% identities with WA, NSW and QLD isolates respectively. The P1 + P2 aa sequence showed greater variation, with the TAS isolate sharing 22% identity with the WA and NSW isolates and 52% identity with the QLD isolate. Amino acid sequence identity for the ORF3 and ORF4 were in the range of 68 – 71% and 64% - 68% respectively.

Table 6.3 Comparison of deduced amino acid sequences for major open reading frames for the Queensland isolate of PhBMYV and some members of the family Luteoviridae

Virus	ORF0 P0 (261 aa) %	ORF1 P1 (581aa) %	ORF1+ORF2 P1 + P2 (1061 aa) %	ORF3 P3 (195 aa) %	ORF4 P4 (143 aa) %	OFR3+ORF5 P3+P5 (656 aa) %
BChV	17	22	16	67	47	32
BMYV	18	27	20	67	49	32
BWYV	20	32	21	68	49	31
CtRLV	13	27	18	54	37	29
CYDV-RPV	14	26	18	66	48	32
CpCSV	18	27	19	69	55	53
CABYV	18	31	20	77	67	48
MABYV	21	29	21	76	70	48
PLRV	14	28	20	65	48	31
ScYLV	11	22	16	39	26	28
TVDV	20	29	20	57	42	30
TuYV	17	29	21	67	48	33
PhBMYV ESPCL15	85	86	60	97	93	34
PhBMYV NSWCP15	76	90	61	95	89	33
PhBMYV TAS 12	97	89	52	71	68	NP
PEMV-1	09	16	08	29	NP	30
BYDV-PAV	NP	07	07	47	28	26
BLRV	NP	09	06	57	44	31
SbDV	NP	08	08	59	46	30

*NP – not present

P1+P2 = RNA-dependent-RNA- polymerase (RdRp)
P3 = Coat protein (CP)
P4 = Movement protein (MP)
P3+P5 = Coat protein + read-through protein (CP-RT)

6.6.4 Phylogenetic analysis

Nucleotide alignment of the complete genome of the new Queensland isolate (QLDCL 16) with the genomes of other Luteoviruses obtained from the Genbank (Table 6.2) showed nucleotide identities in the range of 31 to 88%. PhBMYV isolate QLDCL 16 shared nucleotide identity of 90% and 91% with NSWCP15 and ESPCL15 respectively. Phylogenetic analysis showed the three PhBMYV isolates forming a distinct group (Fig. 4) and appeared closer to MABYV and CABYV (59%), and CpCSV (53%) than the other

Poleroviruses. Phylogenetic analysis of the four PhBMYV (Fig. 6.4B) showed PhBMYV ESPCL 15 and PhBMYV TAS 12 grouping separately from the other isolates.

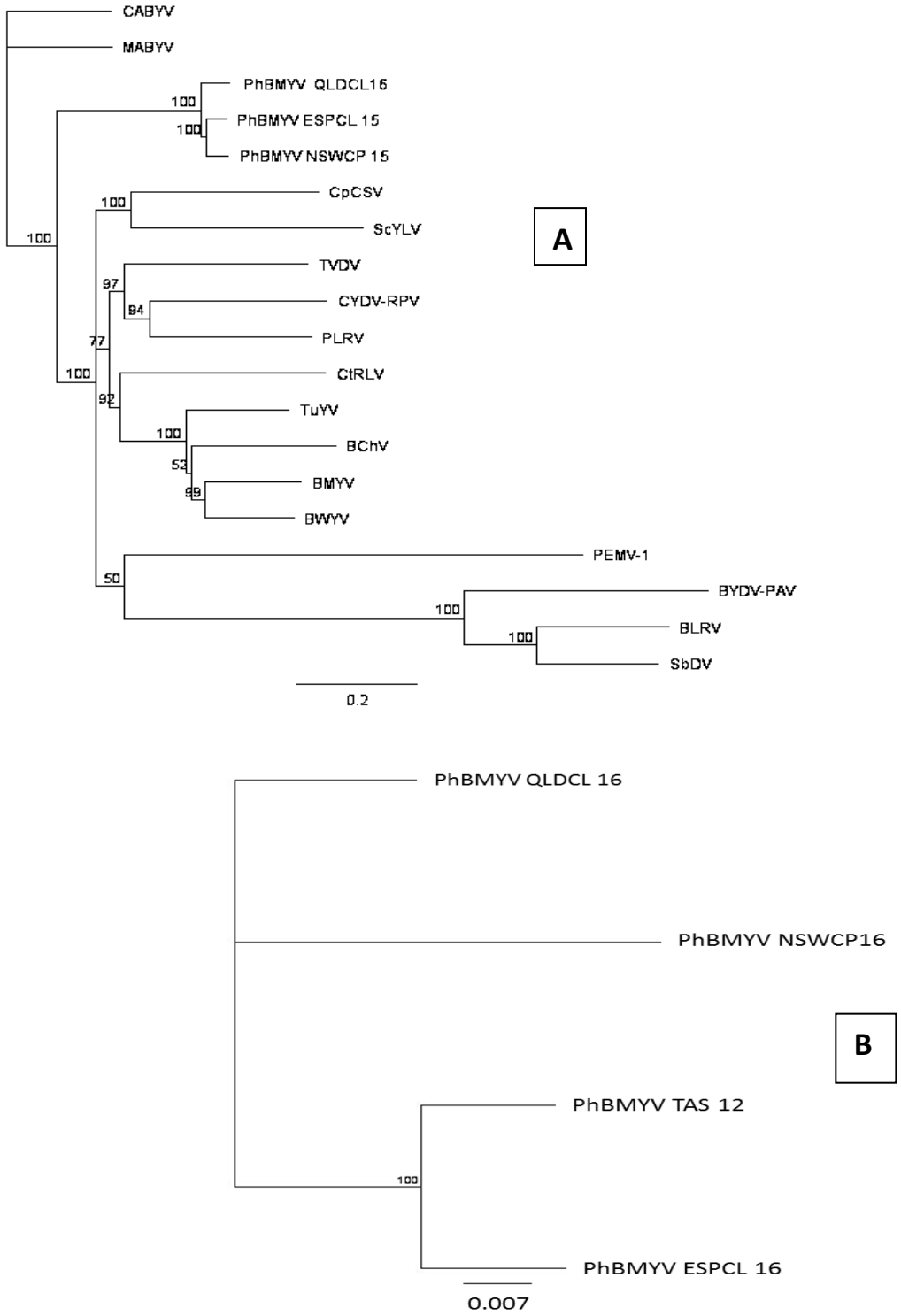
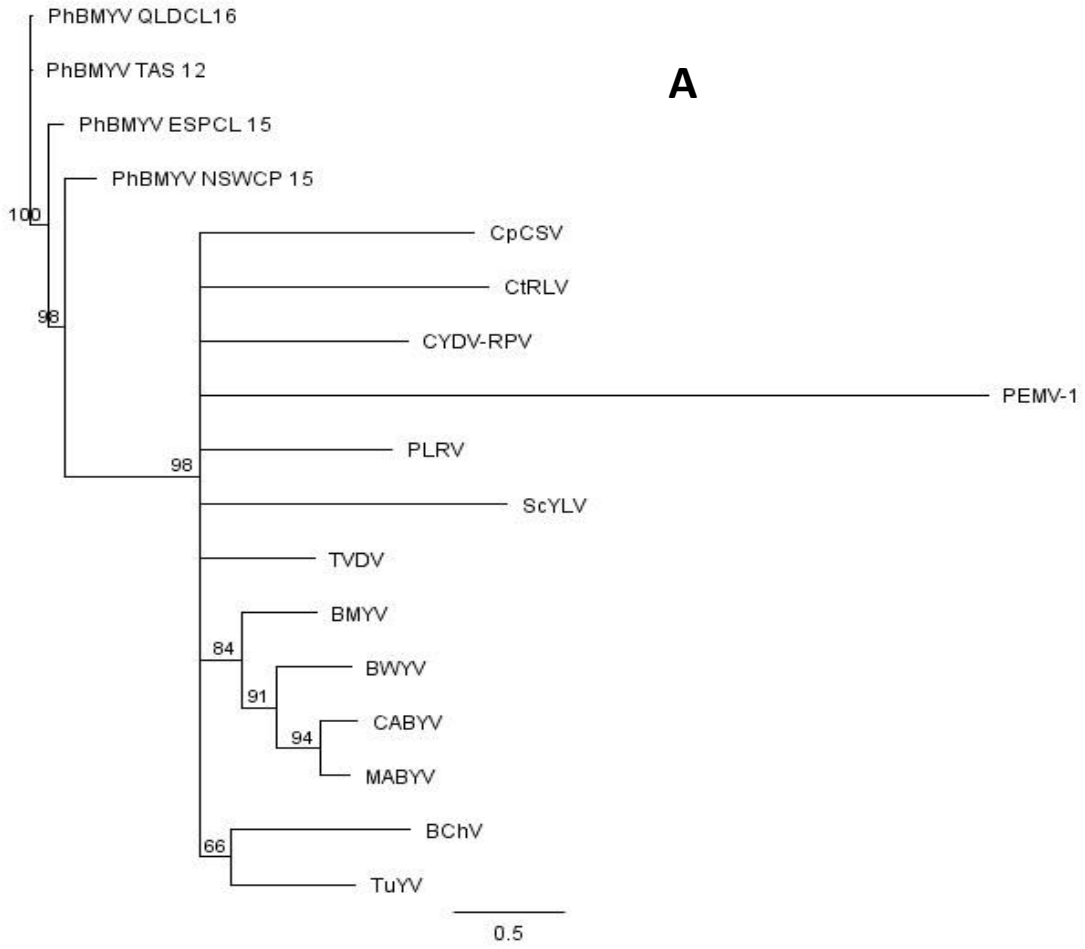
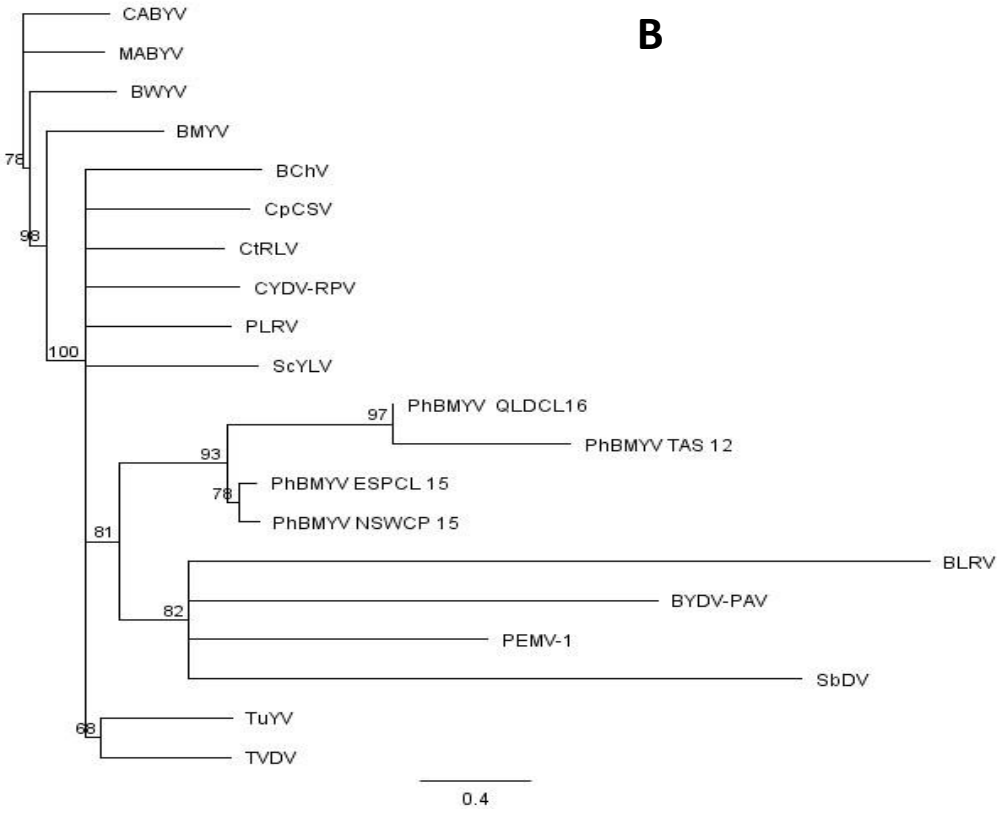
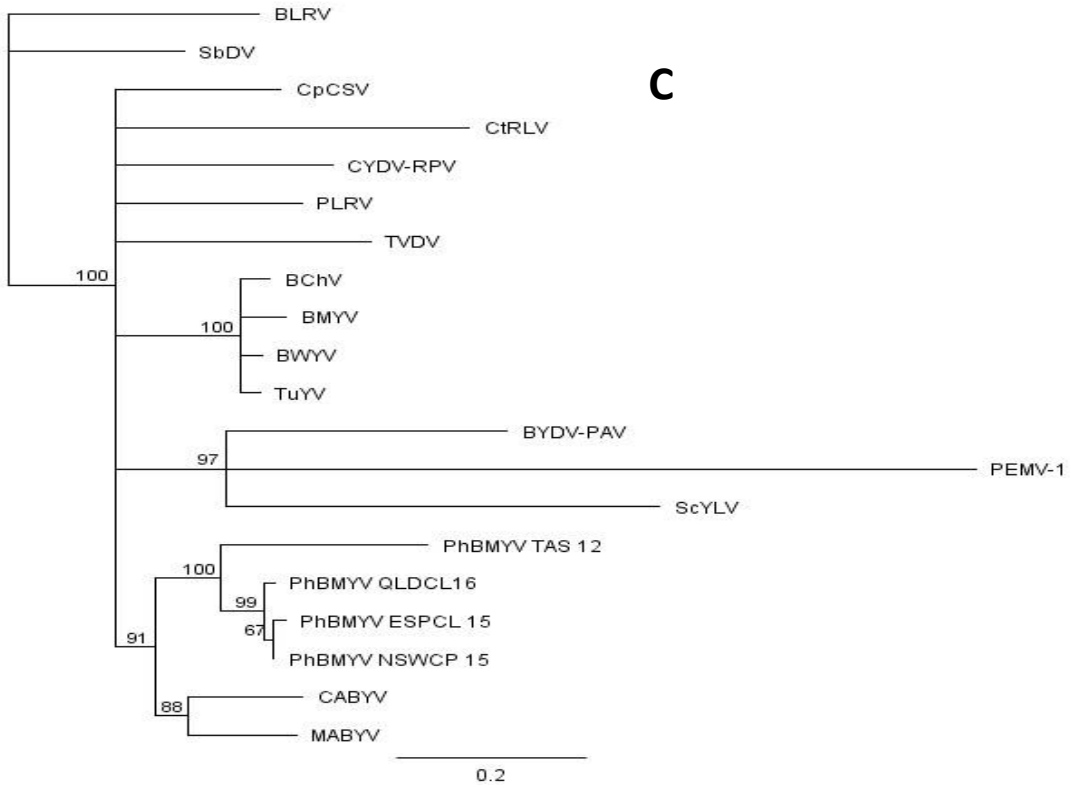


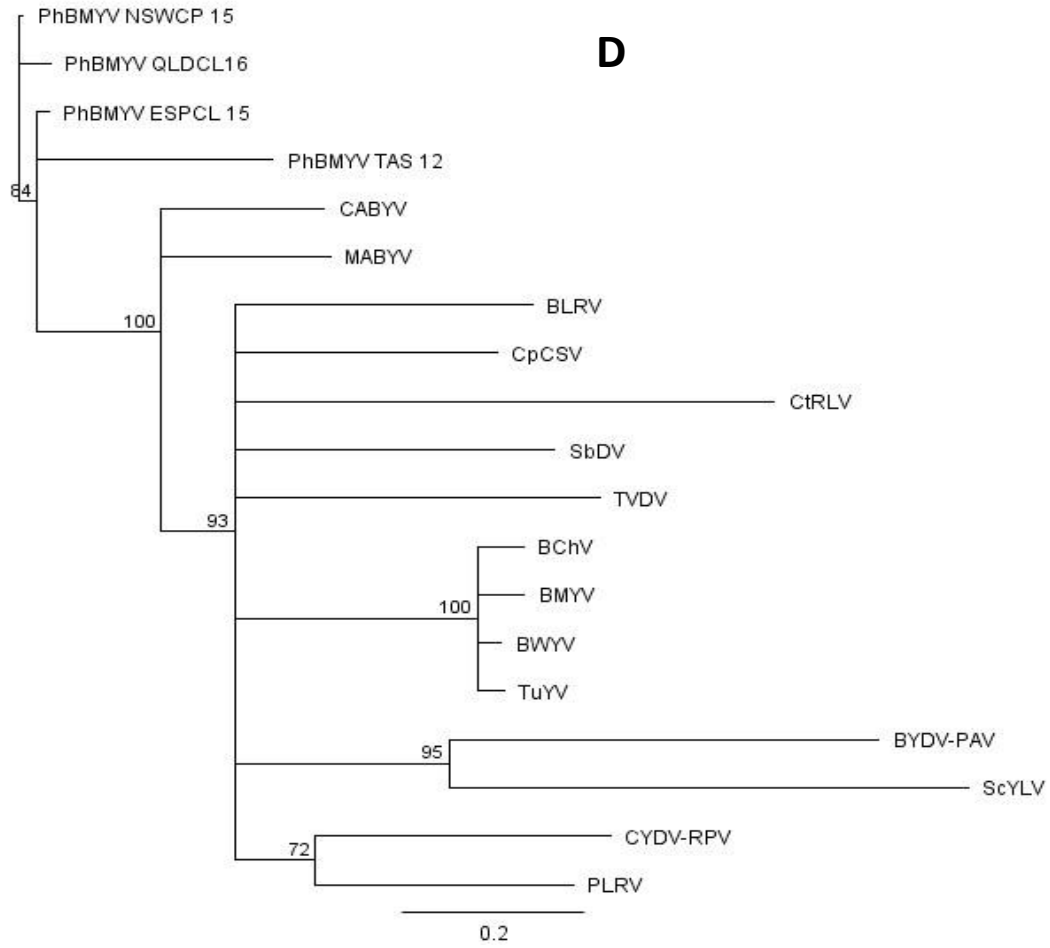
Figure 6.4 Phylogenetic relationship between **A.** PhBMYV QLDCL 16 and other members of the family *Luteoviridae*. Trees were based on the complete genome sequences and distances constructed using the neighbour-joining method. **B.** PhBMYV isolates from Australia. Figures at the nodes indicate the frequency of the cluster after bootstrap analysis (1000 replicates). Nodes with values <50% were collapsed.

Phylogenetic analysis of the predicted amino acid sequences of the P0, P1 + P2, P3, P4 and P3+P5 are shown in figure 6.5. The topologies of the phylogenetic trees for the P0, P1 + P2 (RdRp), P3 (CP) and P4 (MP) show close relationship of the PhBMYV QLD CL16 to those of NSW, WA and TAS, usually clustering together, separate from the other Poleroviruses. The CP-RT protein of PhBMYV QLD CL16 was however, more closely related to CpCSV.




B
CABYV
MABYV
BWYV
BMYV
BChV
CpCSV
CtRLV
CYDV-RPV
PLRV
ScYLV
PhBMYV QLDCL16
PhBMYV TAS 12
PhBMYV ESPCL 15
PhBMYV NSWCP 15
BLRV
BYDV-PAV
PEMV-1
SbDV
TuYV
TVDV
0.4


C
BLRV
SbDV
CpCSV
CtRLV
CYDV-RPV
PLRV
TVDV
BChV
BMYV
BWYV
TuYV
BYDV-PAV
PEMV-1
ScYLV
PhBMYV TAS 12
PhBMYV QLDCL16
PhBMYV ESPCL 15
PhBMYV NSWCP 15
CABYV
MABYV
0.2


D
PhBMYV NSWCP 15
PhBMYV QLDCL16
PhBMYV ESPCL 15
PhBMYV TAS 12
84
CABYV
MABYV
100
BLRV
CpCSV
CtRLV
SbDV
TVDV
93
BChV
100
BMYV
BWYV
TuYV
95
BYDV-PAV
ScYLV
72
CYDV-RPV
PLRV
0.2

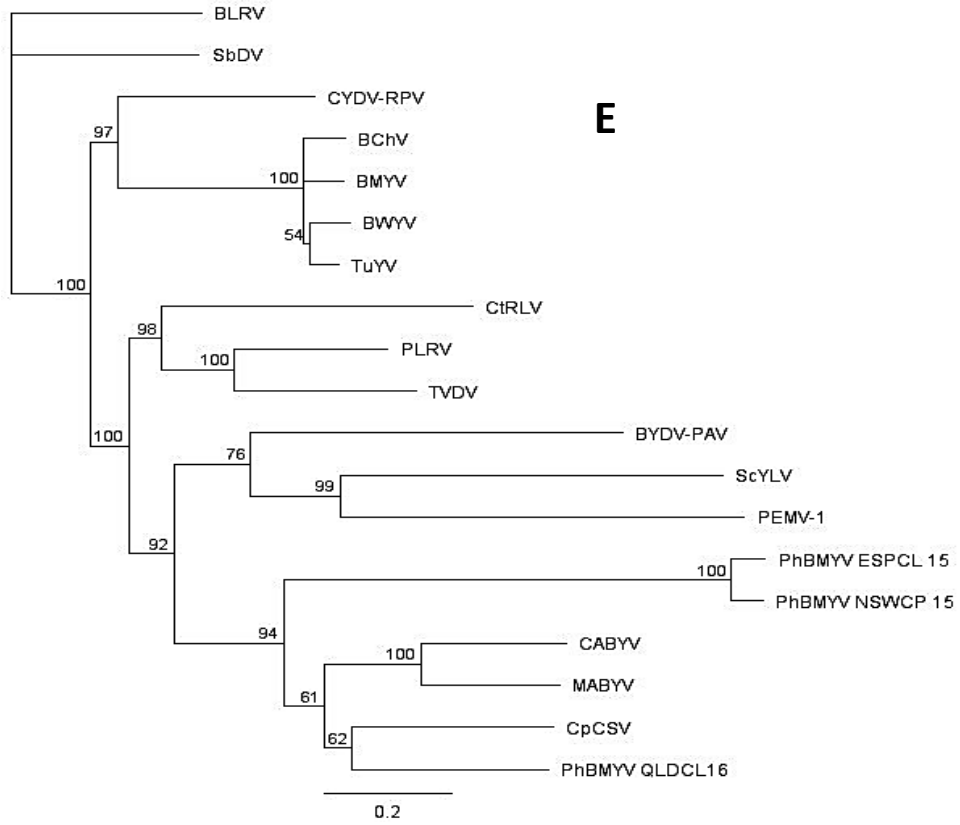

E
BLRV
SbDV
CYDV-RPV
97
BChV
100
BMYV
BWYV
54
TuYV
100
CtRLV
98
PLRV
100
TVDV
100
BYDV-PAV
76
ScYLV
99
PEMV-1
92
100
PhBMYV ESPCL 15
PhBMYV NSWCP 15
94
100
CABYV
MABYV
61
CpCSV
62
PhBMYV QLDCL16
0.2

Figure 6.5 Phylogenetic analysis based on the predicted amino acid sequences **A.** P0, **B.** P1+P2, **C.** P3, **D.** P4 and **E.** P3 + P5. Distance trees were constructed using the neighbour-joining method. Figures at the nodes indicate the frequency of the cluster after bootstrap analysis (100 replicates; shown only when >50%).

6.7 Recombination analysis

Using default settings, the results from RDP suggested PhBMYV QLDCL 16 as a potential evolutionary product of a recombination. (Fig. 6.6). The RDP located the recombination event between positions 5284 and 5975 with a *P* value of 1.825 x10^{-26}. The same event was detected by GENECONV (*P*=2.838 x $10^{-53)}$), BootScan, (*P*= 3.924 x 10^{-35}), MaxChi (*P*=6.752 x10^{-15}), Chimaera (*P*= 6.941 x 10^{-19}), Siscan (*P*= 5.304 x 10^{-52}) and 3Seq (*P*=4.884 x10-^{26}).

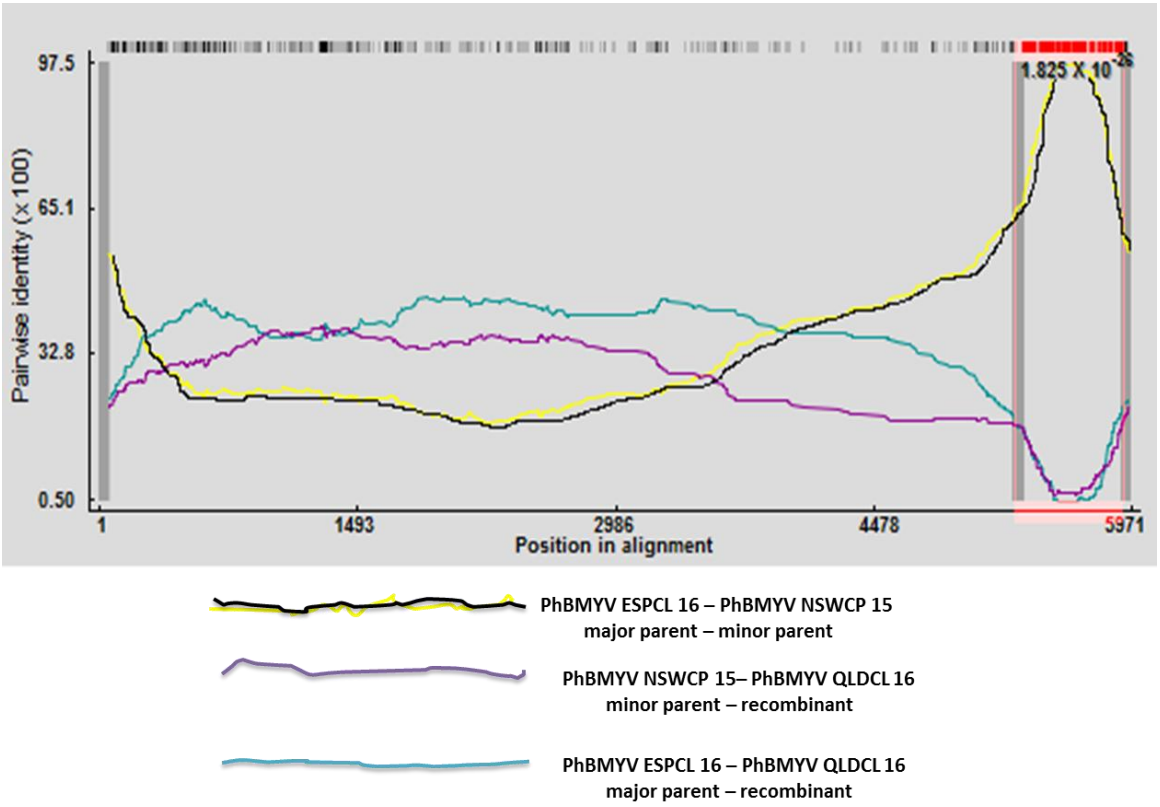


Figure 6.6 A graph showing the result of recombination analysis of aligned Polerovirus genome sequences using the RDP method. The complete nucleotide sequence of the Queensland isolate of Phasey bean mild yellows virus (potential recombinant) was compared to those of Phasey bean mild yellows virus isolates ESPCL 15 (potential major parent) and NSWCP 15 (potential minor parent).

6.8 Discussion

This study reports the full genome sequence of Queenland and a partial genome sequence of Tasmanian isolates of PhBMYV; a novel polerovirus infecting legumes in Australia. The QLD isolate was originally found in Phasey bean; a perennial weed while the TAS isolate was found in peas. Two prior genomes of this virus have previously been reported infecting chickpea in New South Wales and subterranean clover in Western Australia (Sharman *et al*.,

2016). An additional partial genome was reported from field peas in Tasmania (Wilson *et al*., 2012). In this study, successful transmission of the Queensland isolate was achieved to healthy Phasey bean, chickpea and pea plants using both the cowpea aphid vector and grafting. Symptoms expressed in infected Phasey bean plants were severe, contrary to the mild symptoms observed in the field (Sharman *et al*., 2016) and included yellowing, stunting and reduced plant leaf size (Fig. 6.1). Similar severe symptoms were expressed in infected peas and chickpeas. The severe symptom manifestation in the glasshouse could be attributed to the modified environmental conditions especially light and temperature. Reduced illumination has been shown to enhance virus content and increase the susceptibility of plants to infection (Bawden and Roberts, 1947). Furthermore, Manfre *et al*. (2011) demonstrated increased susceptibility of *Nicotiana banthamiana* grown in low light intensity to *Turnip mosaic virus*. In addition, the relatively low average day temperature ($20\pm2^{o}C$) in the glasshouse may have influenced virus replication resulting in high virus concentration in infected plants, leading to severe symptoms as has been reported by Vela´zqueza *et al*., 2010. Attempts to infect other legumes such as cowpea, peanut, Faba beans and members of the Solanaceae (tomato, pepper, and eggplant), Cucurbitaceae (cucumber, zucchini) and Asteraceae (Lettuce) were unsuccessful. The plants remained symptomless and the virus was not detected suggesting they may not be hosts of this new virus. Nonetheless, there is the need to test different varieties of these plants to ascertain differences in varietal response to the virus.

The genome organisation of the PhBMYV QLDCL 16 and PhBMYV TAS 12 were similar to other Poleroviruses, containing six ORFs (except for ORF5 which is yet to be determined for TAS 12 isolate). Genome comparisons revealed close nucleotide identity to PhBMYV ESPCL 15, PhBMYV TAS 12 and PhBMYV NSWCP 15 compared to the other members of the genus, sharing nucleotide identities of >90%. Other Poleroviruses of close identity to this

virus were CABYV and CpCSV. Phylogenetic analysis of the full genomes saw isolate QLDCL 16 clustering with isolates ESPCL 15 and NSWCP 15, forming a distinct clade, but appeared closer to CABYV, MABYV and CpCSV than the other Poleroviruses. This reflects the results of Sharman *et al.* (2016) whom using partial sequences suggested isolates ESPCL15, NSWCP15 and the CABYV-like isolate from Tasmania (HQ543091) are all members of the same PhBMYV, a novel polerovirus. In a separate phylogeny involving the four PhBMYV isolates, isolate TAS 12 appeared closer to ESPCL 15 than NSWCP 15 and QLDCL 16.

With the exception of PhBMYV isolates ESPCL 15, NSWCP 15 and TAS 12, the sequence identity of the predicted amino acids between PhBMYV QLDCL 16 and other Poleroviruses was relatively low and significantly different for all the virus encoded proteins. Although the predicted P3 and P4 proteins of QLDCL 16 differed significantly from those of other Poleroviruses, they appear less variable within the genus. According to Guyader and Ducray (2002) and Plchova *et al*. (2009), ORF 3 (P3) and ORF 4 (P4) are the most conserved regions in PLRV; the type member of the genus. Isolates ESPCL 15, NSWCP 15 and TAS 12 were much closer to QLDCL 16 in amino acid identity for the P0, P1, P3 and P4 proteins, with TAS 12 sharing aa identity of 97% for the P0. Contrary, the predicted amino acid for the coat protein-read through (CP-RT) segment (P3-P5) of the genome was distinct from isolates ESPCL 15 and NSWCP 15, showed closer identity to CpCSV, which we suggest may indicate a recombination event. The P5 protein is involved in aphid transmission of Poleroviruses (Brault *et al*., 2003) therefore, the high variability in the P5 amino acid sequences between the QLD and the NSWCP 15 and ESPCL 15 isolates may suggest that these viruses have different aphid vectors. The predicted P1+P2 fusion protein (RdRp) also differs from isolates ESPCL 15 (60%), NSWCP 15 (61%) and TAS 12 (52%). Phylogenetic

analysis of all virus-encoded proteins saw all three PhBMYV isolates clustering together, except the CP-RT where isolate QLDCL 16 grouped with CpCSV in a separate clade.

With the exception of P1, P3 and P4 proteins, all the other virus-encoded proteins of QLDCL 16 were significantly different from those of ESPCL 15 and NSWCP 15 by more than 10% in amino acid sequence identity. Likewise, isolate TAS 12 differed significantly from isolates ESPCL 15, NSWCP 15 and QLDCL 16 for all virus-encoded proteins by more than 10% except for the P1 and P0 (which showed 97% identity to QLDCL 16). Using the Polerovirus species demarcation criterion of Domier (2011), PhBMYV QLDCL 16 and TAS 12 should be considered distinct strains of the novel PhBMYV, which may have arisen through recombination. Recombination analysis by RDP4 identifies isolate QLDCL 16 as a recombinant; with isolates ESPCL 15 as the major parent but the minor parent is unknown (potentially isolate NSWCP 15). Recombination events have been shown to play important role in generating diversity among members of family *Luteoviridae.* RNA recombination event(s) may have been responsible for the divergence observed between the genera *Luteovirus* and *Polerovirus* (D'Arcy and Mayo, 1997). Subsequent recombination events between polerovirus-polerovirus (Gibba and Cooper, 1995) and luteovirus-polerovirus (Rathjen *et al*., 1994; Moonan *et al*., 2000) have been reported.

The mapping of the complete and partial genomic sequences of the QLD and TAS isolates of this novel virus, development of virus specific primers for its detection and the identification of some potential hosts for isolate QLDCL16 are important steps towards the control and management of the disease caused by this virus. It is however, imperative to ascertain the natural host range by conducting field surveys of legumes of economic importance as well as other plant families known to be infected by Poleroviruses. Experimental host range studies should be expanded to include the vast majority of legumes including pasture legumes which are of economic importance in Australia. Given the wide geographic range of PhBMYV

including tropical, mediteranean and cool temperate climate regions, it is likely that distinct aphid species are involved natural transmission. Further studies on identification of aphid vectors of PhBMYV isolates from around Australia should be conducted. This information is useful especially in management strategies targeting the insect vector. Losses due the disease should be assessed and quantified in field experiments while efforts should be made towards breeding for resistance.

6.9 Acknowledgement

The authors wish to acknowledge the contributions of Dr Alison Dann, Mr Peter Cross, Mr Shane Rossel and Dr Tamilarasan Thangavel. We are also grateful to the Department of Primary Industries, Parks, Water and Environment (DPIPWE) for the use of the TASAG ELISA Lab.

7 CHAPTER SEVEN: Discussion, conclusion and future studies

7.1 General discussion

Plant viruses and viruses-like agents cause disease in most crop plants with effects ranging from latent infections to severe disease including in rare occasions plant death (Gergerich and Dolya, 2006). All major staple crops suffer from at least one debilitating disease induced by virus infection which worsens the current deficit of global food supply where currently at least 800 million people are inadequately fed (Strange and Scott, 2005). Virus diseases are particularly problematic as once infections occur, there are no treatments to mitigate disease and thus control must focus on prevention of infection or genetic tolerance (Strange and Scott, 2005). This study has shown the effect of GRD on nutritive and elemental composition when groundnut plants succumb to the disease. The results indicate drastic changes in nutritive qualities of peanut leaf, stem and seed tissues due to GRD infection. Elevated levels of 3 elements were found in virus infected plants while the levels of 4 elements were inconsistent (Chapter 3). It has identified putatively GRD-resistant groundnut cultivars which could be used in the interim management of the disease and also utilised in breeding programmes (Chapter 4). The study also identified *Groundnut ringspot virus* (GRSV) in Ghanaian groundnuts for the first time (Chapter 4) and assessed the diversity of Ghanaian isolates of *Groundnut rosette assistor virus* (GRAV), *Groundnut rosette virus* (GRV) and the satellite RNA, which are the agents of GRD (Chapter 5). Possible hosts and complete genome sequence of a novel Polerovirus infecting legumes in Australia were also determined (Chapter 6). The work reported herein are not the total solutions to these viral diseases but offer the prerequisite knowledge on which control measures would be formulated and monitored.

7.1.1 Effect of GRD on proximate and elemental composition groundnuts

Several studies have focused on the effect of viral diseases on plant growth with minimal attention on the potential effect on the nutritive value. Although peanuts are a good source of protein for humans and peanut cake serves as a livestock feed, the impact of GRD on the nutritive value of peanuts has not been examined so far. This study (Chapter 3) reports changes in nutrient levels of leaf, stem and seed tissues and the proximate (moisture, ash, toatal carbohydrate, crude protein and fat) content of seeds from GRD-infected groundnut plants. Significant changes due to the disease were observed but these varied with variety. Consistent with the findings of Pazarlar *et al.* (2013), the current study observed reduction in water content from seeds of GRD-infected plants in three of four varieties resulting in shrivelling of seeds. This makes the seeds less attractive and could potentially reduce its market value. Protein content was elevated in seeds of virus-infected plants of cultivars 'Nkosuor' and 'Otuhia' while a reduction was seen in seeds from Sinkapoporigo. Several other reports also note elevation (Singh and Srivastava, 1974; Radwan *et al.*, 2007; Cheema *et al.*, 2003; Sutha *et al.*, 1998) and reduction (Taiwo and Akinjogunla, 2006; Ross *et al.*, 1989) of protein content due to virus infection. Carbohydrate content of virus-infected seeds was inconsistent among the cultivars and this could be due to differential responses to the disease as reported by Kuria *et al*. (2017) and Mandal *et al*. (2002). Moreover, disparities in the carbohydrate content of virus-infected seeds have been reported by different authors (Ross *et al.*, 1989; Mali *et al.*, 2000). Energy content increased in three of the four cultivars, probably due to the increased fat content and it is assumed that this may provide nutritional boost to compensate for any loss in yield resulting from the disease.

Elevated levels of K, Al and Cl were found in all plant parts tested in at least three of the GRD-infected cultivars. Mg, Mn, Ca and Zn did not show any consistent change while Na was decreased in stems but increased in seeds. Increase (Yardinci *et al*., 2007; Shattuck, 1987)

and decrease (Shattuck, 1987; Mofunanya *et al*., 2015; Yardinci *et al*., 2007) in K, Mg and Zn have been associated with virus-infected plant parts. Furthermore, decreased Mn (Yardinci *et al*., 2007), Ca, Na, and Fe (Mofunanya *et al*., 2015) content of virus-infected plant parts have been reported. V and Fe were not detected in seeds but were found at low levels in leaves and stems. Despite these findings, the study did determine the vitamin content and the amino acid profile of the GRD-infected groundnuts. It also failed to determine the level of some important elements such as nickel, cobalt, phosphorus, molybdenum, boron and especially sulphur which plays vital role in disease resistance (Höller *et al*., 2010). Nevertheless, the uncertainty regarding mineral toxicity of GRD-infected groundnuts has been ascertained in at least some of the important minerals.

7.1.2 Screening for resistance to GRD and first report of GRSV in Ghana

Due to the lack of therapeutic measures to cure virus-infected plants, indirect measures are often deployed to control viral diseases. Although aphid-control using insecticide-sprays can be used to minimize the spread of GRD, it is not economically feasible for smallholder farmers in Ghana. Furthermore, improper use of insecticides could lead to the development of insecticide-resistant aphid biotypes, besides human health and environmental risks. Other management strategies such as rogueing, manipulating planting dates and plant density have also been used previously to control GRD but with limited success (Subrahmayam *et al*., 1998), making host plant resistance the best option for disease control. In this study (Chapter 4), field resistance screening led to the identification of four putative GRD-resistant cultivars in four local cultivars while resistance was confirmed in three improved cultivars. These putatively resistant varieties could be used alongside already released GRD-resistant cultivars (Olorunju *et al*., 2001; Subrahmanyam *et al*., 1998) in the management of the disease and also utilised in breeding programmes. The break down of resistance of the improved cultivars in this study re-emphasises the need to characterise isolates of GRD agents (Chapter 5) and

used in virus challenge during breeding. Perhaps these cultivars may have succumbed to different strains of the disease agents not present at where they were bred (Appiah *et al*., 2016; Chapter 4). The study has also identified GRSV in Ghanaian groundnuts, commonly in mixed infection with GRD agents. The virus is a relatively recently described one, regarded as an emerging threat to food crop production (Pappu *et al*., 2009). It is possible that GRSV is a recent introduction or may have been present in Ghanaian groundnuts but has escaped detection due to symptom masking by GRD (Appiah *et al*., 2016; Chapter 4). GRSV in Florida has been identified as resulting from the reassortment of TCSV M RNA (Webster *et al*., 2011). Despite the significant outcomes, this study did not attempt to monitor aphid or thrips vector populations in field trials and also dealt with limited number of groundnut cultivars. The study did not clearly associate seasonal influences in Ghana with greatest GRD incidence, although high incidence was observed in the wet season (Chapter 4).

7.1.3 GRAV incidence and genetic diversity within Ghanaian GRD agents

A high percentage of crop loss is caused by viruses because of their abundant presence in most environments (Adriaenssens and Cowan, 2014). Therefore, early identification of plant viruses is vital to preventing virus spread and formulating control measusres (Akinyemi *et al*., 2016). Additionally, viruses with RNA genomes are known to maintain heterogeneous (quasi-species) and divergent populations (Domingo *et al*., 2012) in susceptible plants, with individual isolates capable of exhibiting distinct epidemiological characteristics. Therefore, with a complex viral disease such as GRD, the likelihood of viral isolates overcoming host plant resistance is higher. Chapter 5 of this thesis looks at the distribution of GRAV in the three northern groundnut-producing regions of Ghana (Northern region, Upper east and Upper west) as well as virus isolate characterization of all GRD agents (GRAV, GRV and Sat RNA). GRD was prevalent in all three regions with two major symptom types; green rosette and isolated cases of chlorotic rosette. GRAV incidence was high in all the three regions,

with no significant regional differences in its distrubtion. Nucleotide and amino acid sequences of Ghanaian GRAV isolates did not show any major variation, and shared close nucleotide identity with previously characterised isolates from Nigeria and Malawi. This presents a good opportunity for the development of transgenic groundnut plants (Deom *et al.,* 2000) which could be used across the entire SSA region. With the exception of isolates GhE3GRV- KX607020 and GhE9GRV- KX607024, the Ghanaian GRV isolates were not different from each other. However, the Ghanaian, Nigerian and Malawian GRV isolates formed different clusters in phylogenetic analysis indicating they are probably genetically distinct. This re-echoes the importance of isolate characterization to ensure that inbred lines are challenged with all possible variants of the viral agents, leading to the development of groundnut cultivars with broad spectrum resistance to GRD. Likewise, the Ghanaian isolates of sat RNA were close in nucleotide sequence identity to each other but were distinct from the Nigerian and Malawian isolates (Deom *et al.,* 2000). Sat RNAs poses some limitations in its use in PDR development (Palukaitis *et al*., 1996), nevertheless, the high degree of variability within the sat RNA could be exploited to determine which nucleotide differences are responsible for the different types of symptoms and the degree of pathogenicity (Deom *et al.,* 2000). One important aspect of GRD epidemiology, which this thesis could not address, is the contribution of alternative hosts in the perprtuation of the disease. The study did not also consider the distribution of GRV and Sat RNA, largely because these could not be tested by ELISA or immuno dot blot, the method of choice for the survey.

7.1. 4 Genomic analysis and molecular phylogeny of Queensland and Tasmanian isolatyes of Phasey bean mild yellows virus

Emerging and re-emerging plant viral diseases pose a serious risk to modern agriculture and food security. However, the development of a plethora of modern techniques has the capacity to diminish the impact of emerging plant diseases by allowing their early detection and

characterization. For effective initiation of strategic control measures, detailed knowledge of emerging plant viral disease outbreaks is of paramount importance. The current (Chapter 6) study sequenced and analysed the complete genome of an isolate of the PhBMYV infecting Phasey bean in Queensland (QLDCL 16); a perennial weed and partial sequence of an isolate infecting peas in Tasmania (TAS 12), Australia. It also achieved the transmission of the isolate QLDCL 16 to peas and chickpeas; important legume crops in Australia and developed specific primers for its detection. Contrary to the mild yellowing symptoms observed on Phasey bean plants in the field, severe yellowing and stunting was associated with experimentally inoculated Phasey bean, pea and chickpea plants. Sequence analysis of isolate QLDCL 16 revealed close nucleotide identities with the the previously reported isolates from New South Wales and Western Australia. However, the amino acid sequence for the RNA-dependent RNA polymerase (RdRP) and the coat protein-read through (CP-RT) segment of the genome were substancially distinct, suggesting that the QLD isolate of the virus may be a genetic recombinant. Variability in the CP-RT region may reflect variation in vector specificity. Recombination analysis identified the QLD isolate as recombinant. Likewise, isolate TAS 12 differed from ESPCL 15, NSWCP 15 and QLDCL 16 in all virus-encoded proteins suggestiong it is also a recombinant. The existence of high genetic diversity within the genus Polerovirus, has been attributed to emergence of new viral diseases in various crops worldwide (Lotos *et al*., 2016) and several new Poleroviruses, arising presumably from recombination have been reported (Abraham *et al*., 2006; Lotos *et al*., 2016; Ellis *et al*., 2013; Distefano *et al*., 2010; Xiang *et al*., 2015; Zhang *et al*., 2014; Wang *et al*., 2016; Xiang *et al*., 2011; Knierim *et al*., 2010; Wilson *et al*., 2012). The outcomes of this thesis warrant further studies on the virus and its strains, natural host range, potential aphid vectors and vector characteristics which were not addressed in this study. Furthermore, the impact of the virus on pea and other legumes is currently unknown. Although no obvious symptoms were

observed from the original field samples, experimentally inoculated plants showed substantial yellowing and reduced growth (Chapter six). Therefore, further study of the impact of this virus and its variants on the various legume hosts is warranted.

7.2 Conclusion

This thesis s provides significant outcomes which could be utilised in the management strategies for GRD (Chapters 3, 4 and 5) in SSA and the emerging PhBMYV disease in Australia (Chapter 6). The most important outcomes include but not limited to:

1. GRD infected groundnut cultivars had significant changes in proximate and elemental composition for nuts, stems and leaves. Protein content was significantly increased in seeds of virus-infected plants while changes in mineral composition were rather inconsistent, except for K, Al and Cl whose contents were elevated in virtually all virus-infected plant parts. Fears regarding mineral toxicity of GRD-infected have been allayed (Chapter 3).
2. Four local groundnut varieties were resistant to GRD during field screening trials. These putatively resistant cultivars could be used in the management of the disease and and also utilised in breeding programmes (Chapter 4). Groundnut ringspot virus was identitied in Ghanaian groundnuts for the first time. This presents a new challenge to the management of viral diseases in groundnut in Ghana and necessitates its inclusion in current breeding programmes (Chapter 4).
3. GRD was prevalent in all three northern regions of Ghana. GRAV incidence in the three regions were not significantly different, an information which will be useful in multilocation trials of inbred lines. Nitrocellulose membrane ELISA was successfully used in GRAV surveys, offering a more rapid, efficient and less expensive method of conducting disease surveys (Chapter 5).

4. Ghanaian isolates of GRAV, GRV and sat RNA were not distinct from each other in terms of nucleotide and amino acid sequences. Ghanaian isolates of GRV and sat RNA were however distinct from those reported in Nigeria and Malawi (Chapter 5).
5. The QLD isolate of PhBMYV is a genetic recombinant of WA and NSW isolates. The virus infected peas and chickpeas in experimental aphid and graft transmission studies and could therefore be regarded as potential hosts. The Tasmanian isolate may also be regarded as a recombinant.

7.3 Future research

This study has detailed the effect of GRD on groundnut nutritional composition (chapter 3). However, the content of other important nutrients such as vitamins was not addressed. Vitamin B_1 especially has been shown to induce systemic acquired resistance (SAR) against plant diseases (Ahn *et al.*, 2005) and it would important to investigate its content in GRD-infected and resistant groundnut cultivars. The determination of the amino acid profile of the GRD-infected groundnuts is recommended in order to ascertain which amino acids are reduced or elevated. The content of some important elements such as nickel, cobalt, phosphorus, molybdenum, boron and especially sulphur which plays vital role in disease resistance should be determined in GRD-infected plants.

Chapter four resulted in the identification of putative resistance to GRD in four Ghanaian local cultivars of groundnut. It must be noted that the current study screened only a limited number of groundnut cultivars and there is the need to screen a wider number of cultivars to identify additional sources of resistance. Other areas of relevance which need further research are the determination of whether the putative resistance found in the four local cultivars is related to the viral agents or the aphid vector. Resistance to *A. craccivora* has been identified in some groundnut breeding lines (Herselman *et al.*, 2004). The determination of the natural

host range of the newly reported GRSV in Ghanaian vegetables as well as the identification of its thrips vector(s) is of paramount importance to the control of the disease. Genetic diversity analysis of GRSV isolates from groundnuts and other possible hosts and the inclusion of the virus in current breeding programmes is warranted.

The current study (Chapter 5) addressed GRD in only the major groundnut-growing regions of Ghana and it is suggested that future studies should include other minor growing areas. These minor growing areas occur in different agro-ecological zones and there is the possibility of existence of different strains of the viral agents. It is also necessary to characterize the diversity of GRD agents in all groundnut-growing regions of SSA in order to enhance resistance breeding. Future studies should include the determination of the complete genome sequences of the GRD agents in Ghana, especially the GRAV which has yet to have its complete genome sequenced. The complete genome sequences of GRV (Taliansky *et al*., 2003a) and sat RNA (Deom *et al*., 2000) have been reported.

Although this study has generated the full genome of the QLD isolate and a partial genomic sequence of TAS isolate of the novel PhBMYV, future studies are required to assess and quantify losses due the disease. Average annual loss of about NZ$ 70m has been reported for *Barley yellow dwarf viruses* (Polerovirus) in Australia and New Zealand alone (Johnstone, 1995). Identification of the aphid vector(s) and natural host range of these novel viruses is of paramount importance. This will contribute significantly towards the management of the disease through vector control while efforts should be made towards breeding for resistance. A live isolate of the TAS isolate is also required to undertake host range studies.

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